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13. ABSTRACT *(Maximum 200*

The objective of this research is to establish whether the secreted phosphoprotein osteopontin (OPN) plays a biological role in the progression of breast carcinoma cells, and to determine the nature of this role, by asking if cell properties and genes associated with malignancy are regulated by OPN. This work makes use of a cell culture model system (the 21T series), which includes three established mammary epithelial cell lines derived from the same patient. 21PT cells are immortal but nontumorigenic in the nude mouse; 21NT are weakly tumorigenic, but non-metastatic; and 21MT-1 are tumorigenic, weakly metastatic.

We have found that all three 21T series cell lines express relatively low basal levels of OPN mRNA and protein, compared to highly malignant, metastatic MDA-MB-435 cells. 21PT and 21NT cells transfected with an OPN-containing expression vector showed increased invasiveness through Matrigel (basement membrane). Both control and OPN-transfected populations of 21PT and 21NT have also been injected into nude mice for *in vivo* tumorigenicity and metastasis assay, as have 21MT-1 and MDA-MB-435 cells as a basis for comparison. The biological effects of OPN are also being examined by exposing the parental cell lines to exogenously added OPN. A trend towards increased growth ability in low serum medium with OPN was seen for 21PT and 21NT, although this has not reached statistical significance. Cell adhesion assay showed affinity for OPN to be associated with degree of malignancy (i.e. MDA-MB-435 > 21MT-1 > 21NT > 21PT). All four parental cell lines showed migration towards OPN. Studies using 21PT and 21NT showed this to be RGD-dependent, directed cell movement (chemotaxis), which was completely blocked by anti-OPN antibody. Possible molecular mechanisms of OPN-induced cell migration and invasion are presently under study. Understanding the functional role of OPN in the malignancy of human breast cancer will be of potential importance not only in the interpretation of prognostic information gained through determination of OPN levels, but also in the future development of therapeutic strategies aimed at blocking OPN effects.

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FOREWORD

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Research involving animals was conducted under the regulations and governance of the Canadian Council on Animal Care (C.C.A.C.), the regulatory agency governing all use of animals for research in Canada. The animal protocol used has been approved by the University of Western Ontario Council on Animal Care (Protocol #95211-9). All hazardous material employed in this research were stored, handled, and disposed of according to all applicable federal, provincial, and local regulations. Ontario Regulation 67/93, made under the Occupational Health and Safety Act for Health Care and Residential Facilities, governs the use and disposal of hazardous materials. The London Regional Cancer Centre and Victoria Hospital Research Institute (now London Health Sciences Centre, Research) are in compliance with this act. We also comply with the stipulations of the University of Western Ontario Biohazardous Agents Registry, which covers biohazards and research using recombinant DNA technology.

Alan Tuck 25/06/97

PI - Signature Date

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THE FUNCTIONAL ROLE OF OSTEOPONTIN IN THE MALIGNANCY OF HUMAN BREAST CANCER

I. GENERAL OBJECTIVE

The goal of this research is to determine the functional role of osteopontin (OPN), a secreted, integrin-binding phosphoprotein, in the malignancy of human breast cancer. Recent evidence detailed below (including a number of clinical studies) strongly implicates OPN in breast cancer. In spite of mounting clinical evidence that increased OPN is associated with worse prognosis in breast cancer patients, the biological role(s) of OPN in progression to malignancy are poorly understood. This information will be important to obtain, as potential utility would lie not only in the interpretation of prognostic information obtained by determination of OPN levels (either plasma or primary tumour), but potentially also in the development of treatment strategies based on blocking the effects of OPN in inducing malignant behaviour of breast carcinoma cells.

Over the past two years, Dr. Tuck has been a member of a multidisciplinary breast cancer research group, based at the London Regional Cancer Centre/London Health Sciences Centre. This team has obtained significant preliminary clinical data to support the overall hypothesis that OPN, as detected in blood and tumours of breast cancer patients, plays an important role in breast cancer (see below). Study of the functional role of OPN in human breast cancer has only just begun. As a new investigator, Dr. Tuck has defined a 3-year research plan to achieve this goal, based on the work here described. The U.S. Army B.C.R.P. "Career Development" Award allows for sufficient protected research time to undertake this project.

II. BACKGROUND

OPN is a secreted phosphoprotein which is expressed in a regulated fashion by a limited number of normal cells and tissues (eg. developing bone, lactating mammary gland, kidney, activated T-cells and macrophages, smooth muscle cells [reviews 1,2]). OPN has also been identified by several groups as a tumour-associated protein (review 3), and its expression has been found to be increased in ras-transformed fibroblasts (4-6), and during multistage carcinogenesis in mouse skin (5). Furthermore, down-regulation of OPN expression in ras-transformed fibroblasts has been shown to decrease their tumorigenicity (7,8), adding weight to the concept that OPN can contribute functionally to the malignancy of cells that express it.

In a study of a limited number of patients, Senger reported 4-10X elevated blood OPN levels from 10 of 13 patients with disseminated carcinomas (9), relative to low plasma levels in 13 patients with other conditions. Included in this small study was plasma from a single breast cancer patient, which showed elevated levels of OPN. No clinical information was provided on any of these patients, and there was no attempt to link OPN plasma levels with severity of disease or prognosis. Since that time, there have been three reports of increased osteopontin expression in human breast cancer (10-12). None of these, however, has examined for an association between level of OPN expression and outcome.

Work performed by our group has shown that elevated plasma OPN (as determined by an antigen capture assay) is associated with the presence of metastatic disease (Singhal et al., 13, Appendix 3). In addition, assessment of OPN levels by immunohistochemistry in primary tumours of lymph node negative patients (in a retrospective study) has shown that increased expression of OPN by the malignant cells (as opposed to the tumor infiltrating macrophages) is associated with poor clinical outcome (Tuck et al., manuscript submitted, 14, Appendix 1).

Preliminary studies involving function of OPN

Despite the accumulation of recent clinical evidence for a role for OPN in breast cancer, experimental analyses of the biological function of the molecule have only begun. A role for OPN in cell adhesion phenomena has been proposed, and there is evidence that this may be mediated in part by binding of an internal GRGDS (gly-arg-gly-asp-ser) amino acid sequence to a cell surface integrin ($\alpha v\beta 3$) (15,16). Work by our group (17-20) has shown that interfering with this RGD sequence of OPN blocks its ability to bind to cells, including human breast cancer cells (19,20). OPN may also bind to other non-integrin cell surface receptors (21). There is evidence that binding of OPN to cell surface receptors may in turn induce a signal transduction pathway via integrin-dependent or independent pathways (22, review 2).

Little is known about how OPN binding may in turn influence behaviour of mammary carcinoma cells. We have shown (23) that three murine mammary carcinoma cell lines (D2HAN series) express levels of OPN RNA considerably higher than levels in virgin mouse mammary gland. We have also found that the metastatic human mammary adenocarcinoma cell line, MDA-MB-435, expresses high levels of OPN, binds to OPN in a cell adhesion assay, and moves towards OPN in a cell migration assay (18-20). Furthermore, both the adhesive and chemotactic properties of OPN may be blocked by RGD peptides or by generation of RGD-deficient mutants (20). Although these studies provide evidence that RGD-mediated events are important in OPN-induced cell adhesion and chemotaxis of some mammary carcinoma cells, the significance of these events to the malignancy of these cells has yet to be determined. Exploration of the

functional role(s) of osteopontin in the malignancy of breast carcinoma cells is thus the focus of the proposed work.

21T series cell lines as a model of breast cancer progression

Band et al. (24) generated a series of breast epithelial cell lines from the same patient, diagnosed with infiltrating and intraductal mammary carcinoma. Permission has been received from Dr. Band to use these cells for the proposed research. These cell lines have been characterized for tumorigenicity and metastatic ability in nude mice. 21PT is immortal, but non-tumorigenic; 21NT is tumorigenic, non-metastatic; and 21MT-1 is metastatic. This series of cell lines has been recently used to demonstrate tumor cell-specific loss of p53 protein in human breast tumor progression (25).

The clinical and experimental data described above provide compelling support for the premise that OPN expression may play a role in the biology of breast carcinoma. What is needed now is an understanding of whether OPN can directly influence the malignancy of breast carcinoma cells, at what stage during progression, and in what manner. The 21T series of cell lines provide a useful model system for the study of influences of OPN on breast epithelial cells at different stages of progression. The present study makes use of this cell culture model to address the questions raised above directly, in a controlled and testable fashion.

III. HYPOTHESIS/PURPOSE

The hypothesis to be tested is that the secreted phosphoprotein OPN plays a biological role in the progression of breast carcinoma cells, and that this role involves the modulation of measurable cell properties and alterations of "malignancy-associated" gene expression. The

purpose of this research is thus to: **1) establish whether OPN plays a biological role in the progression of breast carcinoma cells**, and **2) determine the nature of this role in terms of which cell properties and "malignancy-associated" genes may be regulated by OPN.**

IV. TECHNICAL OBJECTIVES

1. **To determine whether OPN expression is associated with malignancy of established human breast epithelial cell lines.**

2. **To determine whether up-regulation of OPN expression changes the malignant properties of these cells.**

3. **To examine how OPN affects other measurable cell properties associated with malignancy.**

OBJECTIVE 1: To determine whether OPN expression is associated with malignancy of established human breast epithelial cell lines.

METHODS

Cell lines and culture

The 21T series cell lines (21PT, 21NT, 21MT-1) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute). These cells are maintained in culture in α-MEM supplemented with 10% FCS, 2mM L-glutamine (all from GIBCO-BRL/Life Technologies,

Grand Island, NY), insulin (1 μg/ml), epidermal growth factor [EGF] (12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma Chemical, St. Louis, MO) (αHE medium). MDA-MB-435 cells were obtained as a kind gift of Dr. Janet Price (MD Anderson Cancer Center, Houston TX), and are grown in α-MEM with 10% FCS (both from GIBCO-BRL/Life Technologies).

RNA (and DNA) isolation and Northern blot analysis of RNA

Cell pellets were mechanically homogenized (Polytron PT 1200, Brinkmannn Instruments [Canada] Ltd., Mississauga, ON) and RNA extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON), according to the protocol supplied by the manufacturer. The DNA containing fraction was also kept, purified and stored as per the manufacturer's protocol. RNA (10 μg/lane) was run on a 1.1% agarose gel with 6.8% formaldehyde, and capillary-transferred to GeneScreen Plus filters (DuPont Canada Inc., Mississauga, ON). Blots were probed with denatured, oligolabelled [^{32}P]-dCTP cDNA probes, using an oligolabelling kit (Pharmacia, Baie d'Urfe, PQ) according to the procedure provided by the manufacturer, and as previously described (26,27). RNA levels were quantified by densitometry (Phosphorimager SI, Molecular Dynamics, Sunnyvale, CA).

The OPN probe used was the full-length (1493 bp) human OPN c-DNA EcoRI cassette of plasmid OP-10 (28,29). Even loading of lanes was assured by probing blots with a human 18s rRNA probe (p100D9; a kind gift from Dr. D. Denhardt, Rutgers University, NJ) and by examination of 18s and 28s ribosomal bands on the ethidium bromide stained gel.

Western blotting of conditioned media

Conditioned media were prepared by plating cells at $5X10^5$ cells/100 mm plate in regular growth medium and incubating overnight (18 hrs) at 37°C, 5% CO_2. Medium was then removed, and plates were washed X1 with warm, sterile PBS, and X2 with serum-free Opti-MEM (GIBCO-BRL/Life Technologies). Serum-free Opti-MEM was then added at 3 mls/100 mm plate, and plates were incubated 24 hrs at 37°C, 5% CO_2. Following the incubation period, the conditioned medium from each plate was collected, the cell debris spun out, and the supernatant stored at -80°C for later use in Western analysis. Each corresponding plate was trypsinized, and a cell count performed, to allow appropriate correction in loading for cell equivalents.

Protein gel electrophoresis was done by standard SDS-PAGE methods (30), and immunoblotting by the enhanced chemiluminescence system (Amersham Canada, Oakville, Ontario). Conditioned media were concentrated by ultrafiltration in centricon-30 mini-concentrators as per the manufacturer's protocol (Amicon Inc., Beverly, MA). The concentration of total secreted protein was measured by Peterson's modification of the standard Lowry assay (31). The amount of total protein loaded was corrected for cell equivalents based on cell counts at the time of harvesting. Secreted protein was then fractionated on a 12% denaturing SDS-PAGE gel, electrophoretically transferred to nylon membrane using a semi-dry system (Millipore Canada, Mississauga, Ontario, Canada), and detected with biotinylated monoclonal antibody mAb53 (0.2 μg/ml) (17), followed by streptavidin-horseradish peroxidase conjugate (Jackson Immunological Laboratories). The enhanced chemiluminescence detection system (Amersham Corp.) was used to detect immune-reactive bands. Film exposure time was 20 s. Molecular mass markers used were biotinylated protein standards (Bio-Rad Laboratories,

Hercules, California). Immunoreactive bands were quantified using the Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Tumorigenicity and "spontaneous" and "experimental" metastasis assay in nude mice

For tumorigenicity and "spontaneous" metastasis assay, 10^7 cells were injected into a mammary fat pad (first inguinal) of female nude mice at 6 weeks of age. Mice are observed weekly, and will be kept for up to 8-12 months. Tumors will be grown to a maximum size of 7-8 cm^2. At the time of sacrifice, organs (including lungs, heart, liver, spleen, kidneys, bowels, brain) will be fixed and examined for the presence of metastatic disease.

For "experimental" metastasis assay, 10^6 cells were injected intravenously into the tail vein of female nude mice at 6 weeks of age. These mice will be kept for up to 5-6 months, at which time they will be euthanized and examined for presence of metastatic disease as above.

RESULTS

Results of Northern analysis have shown that all three parental 21T series cell lines express relatively low levels of OPN mRNA, compared to highly malignant, metastatic MDA-MB-435 cells (Figure 1). Assay of conditioned medium by Western analysis has shown that the 21T series cell lines also secrete lower levels of OPN protein than MDA-MB-435 cells, with the highest levels of expression in the 21T series seen in the weakly metastatic MT-1 cells. The predominant form of secreted OPN seen in the 21T series cell lines was of high molecular weight (~97kD), most likely representing a conjugated (perhaps by transglutaminase) form (Figure 2).

In order to directly compare "spontaneous" and "experimental" metastatic ability of the

control 21T series cell lines with OPN-transfected cells and the metastatic MDA-MB-435 cells (positive controls), nude mouse injections were delayed until stable transfectants of the 21T series cells were obtained and characterized for OPN expression, such that those expressing the highest levels of OPN could be compared against those transfected with the vector-control. On Feb. 19 and 20, 1997, 30 nude mice were injected i.v. by tail vein (5 mice each group) with: 21PT vector-control transfected cells, a 21PT OPN-transfected pool expressing the highest levels of OPN (see below), 21NT vector-control transfected cells, a 21NT OPN-transfected pool expressing the highest levels of OPN (see below), MT-1 cells (untransfected), and MDA-MB-435 cells. On Feb 26 and 28, 1997, the same series of cell lines (minus the MDA cells) were injected into the mammary fat pads of groups of 5 nude mice (total of 25 nude mice) for tumorigenicity and "spontaneous" metastasis assay. Both groups of mice are presently being monitored, but results not yet available at the time of this report.

DISCUSSION

Baseline expression of OPN by the 21T series of cell lines in culture has been examined at the RNA and protein level, in comparison with MDA-MB-435 cells. Interestingly, the 21T series of cell lines, all of which have been shown to be much less malignant than MDA-MB-435 cells when assayed in nude mice, show much lower levels of basal OPN RNA and protein expression. Furthermore, those of the 21T series that are more malignant (21MT-1 cells > 21NT > 21PT) appear by Western analysis of conditioned medium to secrete higher levels of OPN protein. This also would be consistent with the hypothesis that OPN may be associated with malignancy in this series of cell lines. The fact that the predominant form of OPN in the 21T series is of high molecular weight is also interesting, and would be consistent with the

observations of Beninati et al (32) that transglutaminase catalyzed covalent cross-linking of OPN may occur either to itself to form high molecular weight OPN aggregates, or to fibronectin (and perhaps other extracellular matrix components) to form OPN/fibronectin (or other ECM component) complexes. The relative biological activity of these complexes has not yet been shown in comparison with monomeric OPN. Also of note is that EGF, a growth factor that is essential to the continued propagation of the 21T series cell lines in culture (24), and which is thus included in the culture medium for routine culture, has been shown to itself induce increased cell surface transglutaminase activity in some cell types (33,34), which might at least in part account for the predominance of a high molecular weight (presumably conjugated) form in conditioned medium from the 21T series cell lines in comparison with MDA-MB-435 cells, which are normally cultured in the absence of exogenously added EGF. In other cell types (chondrocytes), EGF is known to increase the phosphorylation status of OPN (35). If this were the case in breast epithelial cells as well, this would be another potential mechanism for post-translational modification to a higher molecular weight form. Finally, the relatively low basal levels of expression of OPN in the 21T series cell lines (21PT and 21NT in particular) makes these cell lines good candidates for assessment of the effects of upregulation of OPN expression on malignancy.

As stated above, studies involving the *in vivo* growth characteristics of these cell lines are ongoing, and are being performed in concert with Task 2e, in order to examine in parallel the relative tumorigenicity and metastatic ability of control 21PT and 21NT cells (vector-control only transfectants) with their OPN-upregulated counterparts (see also below). Although initial nude mouse injections have been performed for both "spontaneous" and "experimental" metastasis assay, results of these studies are not yet available at the time of this report (lag

period for establishment of primary tumors being at least 3-4 months according to previous work of Band et al. [24]).

OBJECTIVE 2: To determine whether up-regulation of OPN expression changes the malignant properties of these cells.

METHODS

Transfections

An expression vector for use in transfection has been generated by cloning the full-length human OPN cDNA (EcoRI fragment of plasmid OP-10, [28]) into plasmid pcDNA3 (Invitrogen Corp., San Diego, CA) at the multiple cloning site between the strong, constitutive CMV immediate early gene enhancer-promoter and the (bovine) growth hormone polyadenylation and transcriptional termination signal sequences (between Not I and Apa I sites). This plasmid also contains the neomycin resistance gene, allowing for selection of stable transfectants in G418-containing medium. The control plasmid used for "vector-only" transfections consisted of the unmodified pcDNA3 plasmid. Transfections were performed using the LIPOFECTIN reagent and the procedure described by the manufacturer (Gibco-BRL/Life Technologies), using 2μg plasmid DNA for every 100μl of OPTI-MEM I in Solution A, and 10μl LIPOFECTIN reagent for every 100μl of OPTI-MEM I in Solution B. Following a 48 hr recovery period, transfected cells were subcultured into αHE medium containing 200 μg/ml (active) G418 (Gibco-BRL/Life Technologies) in order to select out stable transfectants. Plates were incubated until discrete colonies had developed, at which time both pooled populations and cloned transfectants were isolated for expansion in culture and further analysis. Conditioned medium was prepared for

each transfectant population (essentially as described above, but without the need for Centricon concentration) for initial screening by ELISA assay for OPN expression. As the basal level of OPN protein expression by the 21T series parental lines in general was quite low in comparison to that expressed by the transfectants, this proved to be the most efficient method of screening for integration **and expression** of the transfected OPN-containing plasmid DNA (as opposed to Southern analysis of all transfectants). Those transfectant pooled and cloned populations expressing the highest levels of OPN were then chosen for expansion, preparation of RNA, DNA, and cytosolic protein, and eventual injection into nude mice (as above).

ELISA for OPN protein expression by transfected cell populations

Initial screening of transfectants for OPN protein expression was performed by ELISA of conditioned medium, essentially as described previously for plasma (13,36). Briefly, this is a capture ELISA based on high affinity mouse monoclonal (17) and rabbit polyclonal antibodies developed against a recombinant human OPN-GST fusion protein (GST-hOPN) (19) that recognizes native human OPN. Maxisorp immunoplates (Life Technologies, Burlington, Ontario, Canada) were coated with mouse monoclonal anti-OPN antibody mAb53 (100 μl/well, 10μg/ml), then blocked with 1% BSA in ST buffer (0.15M NaCl, 0.01M Tris pH 8.0) with 0.05% Tween 20 (Bio-Rad, Mississauga, Ontario, Canada). The wells were extensively washed with the ST-Tween 20 buffer prior to loading 100 μl of conditioned medium at various dilutions in ST-Tween 20 buffer +1% BSA. The samples were incubated for two hours at 4°C for the primary antigen capture step. Sequential incubations at 37°C of 100μl followed by washing were performed with (a) rabbit anti-OPN antibodies (0.8 μg/ml); (b) biotinylated goat anti-rabbit IgG (1:2000 dilution, Jackson Immunological Laboratories, Inc., West Grove, PA). After washing,

streptavidin conjugated alkaline phosphatase (1:2000, Jackson Immunological Laboratories Inc.) was added for 30 minutes at 37°C. The wells were washed with buffer and 100μl of p-nitrophenyl phosphate (1 mg/ml in 100 mM Tris pH 9.5, 100mM NaCl and 5 mM $MgCl_2$) was added and the signal was allowed to develop at room temperature over 4-6 minutes. The reaction was stopped with 50 μl of 0.2 M Na_2EDTA (pH 8.0). A Bio-Rad plate reader was used to quantify the color signal. Recombinant GST-hOPN fusion protein (19) was used as standard, and background estimated by comparison against equivalent amounts of BSA protein. Internal controls of samples of known OPN concentration were used to normalize OPN values obtained from independent assays.

Northern analysis for OPN RNA expression of transfected cells

Cell pellets of transfected cell populations were mechanically homogenized (Polytron PT 1200, Brinkmann Instruments) and RNA extracted using TRIzol Reagent (Canadian Life Technologies, Inc.) as described above. Total cellular RNA (10 μg/lane) was run on an agarose/formaldehyde gel, transferred to nylon membrane, and probed as described above.

RESULTS

The OPN-containing expression vector, prepared as described above, was used in parallel with control experiments using the unmodified parental plasmid (pcDNA3) for LIPOFECTIN transfections (as described). Successful transfection of 21PT and 21NT cells was achieved, both with the OPN-containing construct and the unmodified pcDNA3 plasmid. Four pooled populations of G418-resistant OPN-transfected PT cells were obtained, designated PT/OPai, PT/OPaii, PT/OPbi, and PT/OPbii. These consisted of combined harvesting of approximately

20 colonies, 25 colonies, 20 colonies, and 20 colonies respectively. Two pools of G418-resistant vector-only control transfectants of 21PT cells were also obtained, designated PT/Ci and PT/Cii. These pools originated from combined harvesting of approximately 70 colonies and 50 colonies respectively. Similarly, four pools of G418-resistant OPN-transfected 21NT cells were obtained, designated NT/OPai, NT/OPaii, NT/OPbi, and NT/OPbii. These consisted of combined harvesting of approximately 45 colonies, 25 colonies, 20 colonies, and 20 colonies respectively. G418-resistant control transfectants of 21NT cells were obtained and designated as NT/Ci and NT/Cii. These consisted of combined harvesting of approximately 20 colonies and 30 colonies respectively. From each of these pools, individual cloned populations were also isolated (7-12 per pool). Despite multiple attempts, transfections of 21MT-1 cells with either the OPN-containing plasmid or the vector control consistently resulted in only small, indistinct colonies which could not be successfully expanded. Combined harvesting of whole plates resulted in cell populations that gradually died off in culture. Thus, although initial low level expansion of transfectants was achieved, these transfectants were unstable and could not be continuously propogated for further analysis.

OPN expression by the transfected cells was assessed firstly by assay of conditioned media by ELISA. Those OPN-transfected pools and clones of 21PT and 21NT cells expressing the highest levels of secreted OPN (by ELISA) are shown in Figure 3, along with representative vector-only transfected controls. Four OPN-transfected cell populations were thus chosen for further study; for 21PT cells, these are: pooled population PT/OPaii and clone 12 derived from Pool PT/OPaii, designated PT/OPaiiC12; for 21NT cells these are: pooled population NT/OPbi and clone 4 derived from pool NT/OPaii, designated NT/OPaiiC4. Both pooled and cloned vector-control transfectant populations consistently showed levels of OPN expression by ELISA

that were barely above background (BSA-only control), such that pools PT/Ci and NT/Ci were arbitrarily chosen for further comparative analysis.

The OPN-transfectant cell populations expressing the highest level of OPN by ELISA assay were also examined by Northern analysis for OPN RNA, in comparison with the vector-only transfected controls. These results are shown in Figure 4. All four of the OPN-transfectant populations showed levels of OPN mRNA expression that were higher than in MDA-MB-435 cells, and many-fold higher than that of the vector-only transfected controls.

The first set of nude mouse injections has been undertaken for assessment of tumorigenicity and "spontaneous" and "experimental" metastatic ability of pool PT/OPaii vs. PT/Ci, pool NT/OPbi vs. NT/Ci, and MT-1 cells (as described above). For "experimental" metastasis assay, MDA-MB-435 cells were included for comparison as an additional (positive) control. Injections of clones PT/OPaiiC12 and NT/OPaiiC4 will follow in the same fashion, and mammary fat pad injections of MDA-MB-435 cells will be included again as a positive control and basis for comparison. Results of the first set of injections are pending at the time of this report.

DISCUSSION

The OPN-containing expression vector was effective in obtaining stable transfectants of both 21PT and 21NT cells. A number of pooled and cloned OPN-transfectant populations (of both 21PT and 21NT) were established, and screening of these transfectants by ELISA has allowed us to select out those expressing the highest levels of OPN protein for further study. High levels of OPN expression were confirmed in these transfectants by Northern analysis for OPN mRNA. Stable control (vector-only) transfectant populations of 21PT and 21NT were also obtained, and consistently express comparatively low levels of OPN protein and RNA.

We have not been able to obtain stable transfectants of 21MT-1 cells using either the experimental or control vectors, despite multiple attempts. There did appear to be initial indistinct colony formation after several weeks in culture, but these colonies could not be successfully expanded in G418-containing medium, suggesting that focal transformation was achieved, but that these transfectants were unstable. 21MT-1 cells are the most progressed of the 21T series, having been derived from a site of metastasis (pleural effusion), and having the most abnormal karyotype of the 21T series (24). It is probable that the markedly abnormal karyotype of 21MT cells is associated with significant chromosomal instability, which may explain our lack of success in obtaining stable transfectants with either the experimental or control vectors. In addition, it is the experience of our lab, and others (24), that 21MT-1 cells have different growth properties than 21PT and 21NT cells. Whereas 21PT and 21NT will grow nicely as monolayers on plastic, 21MT-1 cells tend to form colonies, growing in three-dimensional clusters without forming confluent monolayers. This may in part reflect a requirement of MT-1 cells for neighbouring cells, particularly at early stages of growth. Under the conditions of transfection, where neighbouring cells which have not taken up the transfected vector are dying off, those that have taken up the vector may for other reasons be incapable of getting established (lack of collaborating interaction with viable neighbouring cells). Consistent with this premise is the finding of Band et al. (24) that 21MT-1 cells are under some conditions more fastidious in culture, requiring growth factors or components in serum that are not required by 21PT or 21NT cells.

The lack of stable transfectants of 21MT-1 cells will not significantly affect our ability to address the objectives of this project. We will still be able to assess the ability of OPN to

confer tumorigenicity on non-tumorigenic cells (21PT), and the ability of OPN to confer metastatic ability on tumorigenic, non-metastatic cells (21NT). In addition, we will, and have been able to (see below) assess the ability of OPN to modify measureable cell properties associated with malignancy. If we are unable to obtain stable transfectants of 21MT-1 cells, we will continue to assess the parental line as described in Task 3a, and will continue to use these cells as a basis for comparison as a weakly metastatic cell line expressing low to intermediate basal levels of OPN. In addition, we will continue to use MDA-MB-435 cells as a positive control, representing a highly metastatic breast epithelial cell line expressing high levels of OPN.

As described above, the initial set of nude mouse injections has been performed, although results are not yet available at the time of this report. These studies are ongoing, and results will be included at the time of the next annual report. In the meantime, we have proceeded to examine the transfectant cell lines as outined in Task 3 (see below).

OBJECTIVE 3: To examine how OPN affects other measurable cell properties associated with malignancy.

METHODS

Cell growth in low serum

For assessment of cell growth in low (0.5% FBS) serum vs. growth in medium with 10% FBS, cells were plated in triplicate at 5 X 10^4 cells/plate on 60mm plates. Cells were plated either in αHE with 10%FBS, αHE with 0.5% FBS, or OPTI MEM with 5% FBS plus EGF (12.5 ng/ml) and hydrocortisone (2.8 μM). Triplicate plates per condition were trypsinized and counted on specified days for kinetics analysis.

For comparison of cell growth under conditions of low serum in the presence or absence of OPN, cells were plated on 24 well plates at 5 X 10^3 cells/well in αHE with 0.5% FBS. Duplicate wells were seeded per time point per cell line, either in the presence or absence of hrOPN (50 μg/ml). Cells were allowed to grow for the specified time period, then trysinized and counted for kinetics analysis.

Cell adhesion

Cell adhesion assays were performed essentially as described previously (17-20). Briefly, wells of 96-well polystyrene plates were precoated by overnight incubation with a given concentration of either hrOPN (19), RGD deletion mutant hrOPN (mutOPN) (20), fibronectin (Collaborative Research, Bedford, MA), or BSA (heat-inactivated fraction V; Sigma Chemical Co, St. Louis, MO). Exponentially growing cells were trypsinized and treated with soyabean trypsin inhibitor, washed twice and resuspended in attachment medium (αHE plus 2 mg/ml BSA), and then added to plates at a concentration of 4X10^4 cells/well. Cells were allowed to adhere at 37°C for specified times (range of 30 mins to 4 hours). Nonadherent cells were removed by rinsing twice in phosphate-buffered saline and adherent cells were fixed with 1% gluteraldehyde, stained with hematoxylin, and counted under a microscope.

Cell migration

Cell migration assays were performed essentially as described previously (20), using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6 μg/filter and air dried. The gelatin was rehydrated with 100 μl of serum-free αHE medium at room temperature for 90 min. Lower wells contained 800 μl

of αHE plus 0.1% BSA, with or without test proteins; in some cases, as specified (for "checkerboard" analysis), proteins were added to the upper wells, in order to assess directed cell migration. Cells (5 X 10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated at 37°C; the time of incubation (5hrs) for this series of cell lines was based on preliminary experiments in which optimal time for achieving countable numbers of all four parental cell lines (21PT, 21NT, 21MT-1, MDA-MB-435) was determined. At the end of the specified incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with gluteraldehyde and stained with hematoxylin. Cells that had not migrated and were attached to the upper surface of the filters were removed from the filters with wet Q-tips. The lower surfaces of the filters were examined microscopically under 100X magnification and representative areas were counted to determine the number of cells that had migrated through the filters. For 21PT and 21NT cells, experiments were conducted to assess the OPN-specificity of the induced migratory response, in either the presence or absence of anti-OPN antibody in the lower chamber (20 μg/ml anti-OPN antibody, mAb53 [17]). In order to assess for integrin-dependence of the migratory response, other experiments were conducted either in the presence of RGD deletion mutant hrOPN (20) in place of unmodified hrOPN in the lower chamber, or with unmodified hrOPN along with 100 μM GRGDS peptide (20) added to the lower chamber. Finally, the migration responses of 21PT and 21 NT cells to EGF (12.5 ng/ml) and human hepatocyte growth factor (HGF, 10 ng/ml, Sigma) were also assessed, by performing the migration assay in α-medium with hydrocortisone and 0.1% BSA, in either the presence or absence of each growth factor. The ability of hrOPN to effect cell migration either alone or in combination with EGF or HGF was also assessed by performing these assays in the presence or absence of 50 μg/ml hrOPN.

Cell invasion

In vitro invasiveness through Matrigel was assayed as described previously (27), using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA), coated with 35 μg Matrigel (Collaborative Research Inc., Bedford, MA) per filter. The Matrigel concentration was determined by preliminary experiments using MDA-MB-435 cells and representative OPN-transfected 21T series cell lines. Matrigel was diluted to the desired final concentration with cold, sterile, distilled water, applied to the filters, dried overnight in a tissue culture hood, and reconstituted the following morning with serum-free αHE medium. Cells for the assay were trypsinized and seeded to the upper chamber at 5 X 10^4 cells per well in serum-free αHE medium containing 0.1% BSA. The lower chamber was filled with serum-free culture medium with 0.1% BSA and either 5 μg fibronectin (for assays involving transfectants) or 50 or 100 μg hrOPN (for assays of parental (non-transfected) cell lines). Plates were incubated for 72 hours in a 5% CO_2 incubator at 37°C. Following incubation, the upper wells were removed and inverted, fixed with 1% gluteraldehyde in phosphate-buffered saline, stained with hematoxylin, dipped briefly in 1% ammonium hydroxide, and washed with water. The cells and Matrigel were then wiped off the upper surface of each filter with a cotton swab. After air-drying, cells from various areas of the lower surface of the filters were counted under X100 magnification.

All cell adhesion, migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using the Mann-Whitney test, *t*-test, or ANOVA, using SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

Expression of "malignancy-associated" genes in 21T series cells, baseline vs. OPN-induced

Near-confluent (85-90%) cell cultures of 21PT, 21NT, 21MT-1 and MDA-MB-435 cells were incubated overnight (18 hours) in serum-free αHE medium (with 0.1% BSA) in either the presence or absence of 50 μg/ml hrOPN. Cells were then trypsinized, counted, and washed in PBS. Total cellular RNA was then prepared by the Trizol procedure. Similarly, total cellular RNA was prepared from 21T series cell lines stably transfected with either the OPN-containing vector (PT/OPaiiPool, PT/OPaiiC12, NT/OPbiPool, NT/OPaiiC4) or control vector (PT/CiPool, NT/CiPool). Northern blot analysis (electrophoresis, transfer, and probing as described above) for RNA expression of a number of "malignancy-associated" genes is underway. In each case, the previous probe is removed by washing 6-8X in boiling 0.1X SSC/0.1%SDS. The initial set of probes include: OPN (^{32}P oligolabelled full-length (1493 bp) human OPN c-DNA EcoRI cassette of plasmid OP-10 [28,29]), EGF receptor (EGFR) (^{32}P oligolabelled 1.65 kb EcoRI/ClaI cassette of plasmid hEGF-R (human EGF-R in Bluescript KS$^-$[37]), hepatocyte growth factor (HGF) (^{32}P oligolabelled 540 bp BamHI-XhoI fragment of human HGF cDNA [38]), and HGF receptor (HGFR) (800bp EcoRI-EcoRV fragment of the human *met* c-DNA [39]). These will be followed by a series of probes for various secreted proteases (metalloproteinases, cathepsins) and protease inhibitors (TIMPs).

Protein extracts were prepared by lysis in boiling buffer (pH 7.4; 10 mM Tris; 1% SDS; 2 μg/ml leupeptin; 1 μg/ml pepstatin; 0.5 mM phenylmethylsulfonyl fluoride), followed by shearing through a 26 guage needle. After centrifugation at 16,000 xg to precipitate insoluble material, each supernatant was removed, the protein concentration was determined (as described above), and samples were frozen for later use for Western blotting.

RESULTS

Results of serum dependence testing of the 21T series parental cell lines is shown in Figure 5. 21PT, 21NT, and 21MT-1 all showed poor growth in low-serum (0.5% FBS) medium. Lower concentrations of serum could be used to obtain near-normal growth kinetics if cells were grown in OPTI MEM (5% FBS, with EGF and hydrocortisone). Figure 6 shows the results of testing of the effects of adding exogenous hrOPN (50 μg/ml) to low-serum media on growth kinetics of the 21T series parental cell lines, as well as MDA-MB-435 cells. Although there is a trend towards better growth of 21PT and 21NT cells in the presence of OPN, this has not as of yet proved to be a statistically significant difference. At later time points (14 days), all three 21T series cells showed a trend towards higher cell numbers in medium with OPN than without, although again these differences have not yet been proven to be statistically significant. MDA-MB-435 cells have shown no such trend towards improved growth in the presence of exogenous OPN, although as we have shown (see above), these cells make and secrete relatively high levels of their own (endogenous) OPN. Further experiments are planned to increase numbers of observations at specific time points, in order to determine whether the trends seen for cell growth inducing effects of OPN on 21PT, 21NT, and 21 MT-1 are indeed significant.

Cell adhesion studies have shown that all of the 21T series cell lines bind to OPN, although with lesser avidity than MDA-MB-435 cells (Figure 7). Preliminary experiments using varying concentrations of hrOPN at a fixed time point showed optimal binding in the range of 10-50 μg/ml. At 10 μg/ml hrOPN and a 2 hour incubation time, cell attachment was seen in the following hierarchy: MDA-MB-435 > 21MT-1 > 21NT > 21PT. One-way ANOVA (Student-Newman-Keuls method) showed all of these differences to be significant at the $p < 0.05$ level.

The rank order for cell adhesion ability to OPN thus corresponded to rank order of malignancy of each of these cell lines. Use of the RGD deletion mutant hrOPN protein in this assay resulted in only background levels of binding, indicating that the cell adhesion seen with intact hrOPN is integrin-dependent (Figure 7).

Migration assays have shown a significant response of all of the cell lines tested to hrOPN (Figure 8). Preliminary testing at different time points with a fixed amount of hrOPN in the lower chamber established 5 hours as the optimum time point for assessment of all of these cell lines (data not shown). As the average size and shape of 21MT-1 and MDA-MB-435 cells (larger cell size, polygonal > spindled) is markedly different than that of 21PT and 21NT cells (smaller cell size, spindled > polygonal), direct comparisons between these cell lines for migration through a fixed (8μm) pore-size are not generally informative (with the exception of 21PT and 21NT; see below). However, what is apparent is that all four cell lines show significant migration response to 50 μg/ml hrOPN ($p < 0.05$ for all, student's *t*-test). If one focuses on 21PT and 21NT cells, which are of very similar cell size and shape, it is apparent that 21NT shows a higher basal level of migration than 21PT ($p = 0.0003$, student's *t*-test), although both cell lines show significantly increased migration in response to hrOPN ($p = 0.0002$ for both, student's *t*-test). Testing of 21PT and 21NT cells for response to different concentrations of hrOPN showed no significant difference between response to 50 vs. 100 μg/ml hrOPN (Figure 9). Positive control experiments using native bovine bone OPN as the chemoattractant have also been performed, and the cells show a similar (slightly greater in the case of 21NT cells) response to the native protein (data not shown). "Checkerboard" (or "criss-cross") analysis showed the migration response to be predominantly directed cell movement (hence chemotaxis vs. chemokinesis) for both 21PT and 21NT, although 21NT did also show

a minor component of random motility (chemokinetic) response (Figure 10). Addition of blocking anti-OPN antibody to the lower chamber completely eliminated the migration response to hrOPN (Figure 11). Addition of blocking GRGDS peptide to the lower chamber also completely blocked the migration response to hrOPN, and use of RGD-deletion mutant hrOPN in place of unmodified hrOPN resulted in a complete lack of an induced migration response (Figure 12). Interestingly, eliminating EGF from the culture medium was found to reduce basal migration rate of both 21PT and 21NT (Figure 13). Adding hrOPN or EGF alone increased the migration of both cell lines, while adding both together showed a synergistic effect on cell migration (Figure 13). Similarly, hrOPN was also found to enhance the induction of 21PT and 21NT cell migration by human HGF (Figure 14). Assays performed to examine the ability of the parental 21T series cell lines, and MDA-MB-435 cells to invade through basement membrane (Matrigel) in response to exogenous (hr)OPN similarly showed an inductive effect of OPN on all four cell lines (all show $p<0.05$ by student's *t*-test) (Figure 15). To date, a representative control transfectant pool, and the OPN-transfectant pool expressing the highest levels of OPN for both 21PT and 21NT-derived cell populations have been tested for invasiveness through Matrigel (35 μg/well) in the transwell assay. Both OPN-transfected 21PT cells (PT/OPaii) and OPN-transfected 21NT cells (NT/OPbi) were found to show increased invasiveness over the pooled populations transfected with the control vector alone (PT/Ci, NT/Ci) ($p=0.0014$, 0.0312 respectively by the student's *t*-test) (Figure 16). OPN-transfected clones PT/OPaiiC12 and NT/OPaiiC4 have not yet been tested in this assay.

Studies to assess expression of certain "malignancy-associated" genes in 21PT, 21NT, 21MT-1 and MDA-MB-435 cells induced by overnight incubation with 50 μg/ml hrOPN (vs. uninduced) have recently begun. Northern analysis is underway for expression of EGFR, HGF,

and HGFR mRNA (as described in Methods). The next series of probes will examine the expression of several proteases and protease inhibitors believed to be involved in the malignancy of human breast cancer. Similar screening of the transfectant cell populations for expression of the same set of "malignancy-associated" genes will also be shortly underway.

DISCUSSION

Testing of 21PT, 21NT and 21MT-1 cells for culture growth in low serum (0.5% FBS) showed all four of these cell lines to be serum-dependent (Figure 5). Addition of exogenous hrOPN to the culture medium has shown a trend towards increased cell growth in low serum for 21PT and 21NT (Figure 6), although these differences have not yet been proven to be statistically significant. In addition, at later time points (14 days), all three 21T series cell lines (including MT-1 cells) show a trend towards higher cell numbers in low serum medium with hrOPN than without (Figure 6), although again these differences have not yet been proven to be statistically significant. Further experiments are thus planned to increase numbers of observations at specific time points, in order to determine whether the trends towards increased cell growth of 21PT, 21NT, and 21 MT-1 in low serum medium containing hrOPN are indeed significant. Interestingly, MDA-MB-435 cells have shown no such trend towards improved growth in low serum medium in the presence of exogenous (hr)OPN (Figure 6). This could be related to the fact that these cells already make and secrete relatively high levels of their own (endogenous) OPN (see Figures 1,2).

Cell adhesion studies have shown a range of cell binding to OPN, with MDA-MB-435 > 21MT-1 > 21NT > 21PT (10 μg/ml OPN, 2 hour incubation, Figure 7). The rank order for cell adhesion to OPN thus corresponds to the rank order of malignancy of each of these cell

lines. This could be due to increased receptor numbers, or increased affinity of receptors for OPN in the more malignant cell lines. Use of the RGD deletion mutant hrOPN protein in this assay resulted in only background levels of cell binding, indicating that the cell adhesion seen with intact hrOPN is integrin dependent (Figure 7). Previous studies examining the adhesion of other cell types (eg. osteoclasts, osteosarcoma cells, smooth muscle cells, endothelial cells) to OPN have indicated the involvement of integrin receptors $\alpha v\beta 3$, $\alpha v\beta 1$ and $\alpha v\beta 5$ (2,40,41), and in some instances non-integrin receptors such as CD44 (21,42). In our studies, as only background binding is seen when RGD deletion mutant hrOPN is used in the adhesion assays, we are not able to detect a significant RGD-independent (i.e. non-integrin) binding of any of the four cell lines tested. Further work will examine the nature of the adhesion interaction involved for these cell lines, using blocking antibodies to the various different cell surface receptors believed to be involved.

Experiments to assess cell migration response to hrOPN have shown a significant response of all of the breast epithelial cell lines tested (21PT, 21NT, 21MT-1, MDA-MB-435) (Figure 8). Because of differences in cell size and shape, 21MT-1 and MDA-MB-435 cells cannot be compared directly against each other, or against 21PT or 21NT in this cell migration assay (because of the restriction of a fixed pore size). Comparisons between 21PT and 21NT are possible, however, as both are of the same basic cell morphology (spindle shaped, less than 8 μm in narrowest dimension). If one then focuses on these two cell lines, 21NT, the tumorigenic line, generally shows a higher basal migration that its non-tumorigenic counterpart, 21PT (Figure 9, $p=0.0003$ student's *t*-test). Both cell lines, however, respond in a similar fashion by showing increased migration in response to OPN, and "checkerboard" analysis has shown this to be predominantly **directed** cell migration (ie. **chemotaxis** rather than random

migration or chemokinesis) (Figure 10). The specificity of this phenomenon was confirmed by showing complete blocking of induced migration with anti-OPN antibody (Figure 11). The RGD-dependence of the induced migration was proven in experiments using blocking RGD peptide, and RGD deletion mutant OPN (Figure 12). Thus, the migration reponse of the 21T series cell lines to OPN appears to be integrin-dependent (as previously shown for MDA-MB-435 cells [20]). Senger and Perruzzi (43) have recently shown that MDA-MB-435s cells (and human T24 bladder carcinoma cells) migrate in response to a GRGDS-containing thrombin-cleavage fragment of osteopontin, and that this migration is primarily $\alpha v\beta 3$ integrin-dependent. For this reason, and for the reasons described above related to previously documented binding interactions of OPN with cell surface receptors of other cell types, we are now interested in identifying the specific receptors involved in the 21T series migration response to OPN as well, using blocking antibodies as described above in relation to cell adhesion inhibition. Finally, and perhaps most importantly, we have found evidence for a synergistic interaction between OPN and growth factors EGF and HGF in inducing the migration of 21PT and 21NT cells (Figures 13,14). Similar synergistic interactions beween growth factors and other integrin-binding proteins have been recently reported, and potential pathways for "cross-talk" between signal transduction pathways activated by these two different (but perhaps convergent) routes have been suggested (reviews in 44-48). Once the cell surface receptors bound by OPN are identified, further exploration of specific signal transduction components/pathways involved in OPN induction of cell migration would thus be warrented, with the emphasis on potential for "cross-talk" with pathways which have been described for EGF and HGF.

Invasion assays using exogenous (hr)OPN as a chemoattractant have similarly shown 21PT, 21NT, 21MT-1 and MDA-MB-435 cells all to respond to OPN. All four cell lines

showed significantly increased invasion through Matrigel (35μg, 72 hr assay) when 100 μg/ml hrOPN was present in the lower chamber (Figure 15) ($p<0.05$ by student's *t*-test for all). (This response was not apparent at 50 μg/ml hrOPN, data not shown). The OPN-transfected pool of 21PT cells and 21NT cells expressing the highest level of OPN protein (as determined by ELISA assay) (PT/OPaii, NT/OPbi) have also been tested for invasion in response to fibronectin as the chemoattractant, and found to show increased invasiveness over the pooled populations transfected with the control vector (PT/Ci, NT/Ci) (Figure 16) ($p=0.0014$, 0.0312 respectively, student's *t*-test). Experiments are planned to examine OPN-transfected clones PT/OPaiiC12 and NT/OPaiiC4 in this assay as well. Although again, due to marked differences in cell size and shape between the parental cell lines, direct comparisons of invasiveness between 21MT-1 and MDA-MB-435 with 21PT and 21NT cannot be made, it is significant that all four do respond to hrOPN by increased invasiveness in this assay. Thus, even at the earliest stage of tumor progression (established, non-tumorigenic 21PT cells), breast epithelial cells are capable of responding to OPN by increased invasiveness through basement membrane. This evidence is borne out by the stable transfectants, which also showed increased invasiveness in the presence of constitutive expression of transfected, native OPN by the cells themselves. It would appear then, that the presence of OPN could thus influence malignancy even at quite early stages of malignancy and that the differences in *in vivo* malignant behavior of the 21T series cells and MDA-MB-435 cells may be related to their relative ability to independently express OPN, or to their relative affinity for OPN (as cell adhesion assays have shown a relationship between maligancy and OPN binding of these cell lines). These data would thus fit well with our recently completed study of lymph node negative breast cancer patients, which has shown that whether it is synthesized by the cancer cells themselves, or taken up from the environment, it

is the OPN present in breast cancer cells themselves (as opposed to that present in tumor-infiltrating macrophages and lymphocytes) which appears to be related to prognosis (see Appendix 1).

RECOMMENDATIONS

This project is proceeding as outlined in the statement of work, with good progress, much of which should soon be publishable. Two recommendations regarding the statement of work should here be made. Regarding the *in vivo* testing in nude mice, it would be recommended that the time course for these assays be extended over the full three years of the award. The 21T series cell lines require long incubation periods for tumorigenesis to occur (at least 3-4 months following mfp injection [24]), such that there are long intervals between time of injection and collection and analysis of results. In order to optimize numbers of mice used in these experiments, it is best not to inject and follow more than about 50 mice at a time (as described, we are presently following 55 injected mice). Adjustments in the injection regimen may be required based on the results of this initial set. Further, if significant effect is seen in small groups of mice (five per treatment group), it may not be necessary to use the full 10 mice/group initially proposed.

Regarding the use of 21MT-1 cells, we would propose that these cells best be used as a positive control (along with MDA-MB-435 cells) for *in vivo* and gene expression assays. Attempts to transfect these cells have been unsuccessful, most likely due to the high degree of aneuploidy and associated genetic instablity of this line. Further, these cells cannot be directly compared against 21PT and 21NT in cell migration and invasion assays, due to their marked difference in size and shape. In contrast, 21PT and 21NT are much more amenable to direct

comparison in these assays, due to their very similar cell size and shape. Particular focus on these two cell lines has proven quite fruitful, and we would propose to continue to emphasize these two cell lines (and their transfected counterparts) in future work.

We would propose that further work involving 21PT and 21NT should include the identification of the cell surface receptor(s) responsible for the induced cell migration effect of OPN. Use of blocking antibody to different cell surface integrins (including αvß3, αvß5 and ß1) would be made to screen for involvement of integrins known or suspected to bind OPN. Anti-CD44 antibodies could also be used to determine if blocking of this transmembrane receptor effects OPN function in inducing cell motility as well. As described above, further work will also examine the ability of OPN to effect the expression of other genes known to be involved in cell motility and invasiveness.

CONCLUSIONS

Work to this point has revealed a number of potential biological functions for OPN in the malignancy of human breast cancer. Using the model system of the 21T series cell lines, along with the highly metastatic MDA-MB-435 cells, we have shown that the more malignant cells of this series not only secrete higher levels of OPN protein, but bind better to OPN in cell adhesion assays (i.e. MDA-MB-435 > MT-1 > 21NT > 21PT). The latter would suggest either higher numbers of appropriate cell surface receptors for OPN on the more malignant cells, or a higher affinity/availability of those present for OPN binding. Further work will examine which cell surface receptor(s) are involved in this adhesion, although we have some evidence from work with the RGD-deletion mutant OPN that it is RGD- (and hence integrin) dependent.

In addition, we have shown that all of the breast epithelial cell lines tested (21PT, 21NT, 21MT-1, and MDA-MB-435 cells) respond to OPN by increased cell migration and increased invasion through Matrigel (basement membrane). In more detailed examination of 21NT and 21PT cells, we have shown that although 21NT cells show a higher basal level of migratory activity, both 21NT and 21PT are capable of responding to OPN by increased migration in a directed (chemo/haptotaxic) fashion. That the induced cell migration was specific was shown by complete blocking with anti-OPN antibody; and RGD-dependence of the phenomenon was demonstrated by complete blocking with GRGDS peptides and lack of response to RGD deletion mutant OPN. As is the case for cell adhesion, the cell migration response thus appears also to be integrin-dependent, and again further work will be aimed at identifying the specific cell surface receptor interaction involved. A synergistic relationship between OPN and EGF and HGF in inducing cell motility has also been identified through this work. Studies are underway to determine whether OPN can itself influence the expression of EGFR, HGFR (MET) or HGF in these cell lines.

The increased cell invasiveness (through Matrigel) in response to OPN was seen both using exogenous (hr)OPN, and by assessment of pools of transfected (21PT and 21NT) cells stably expressing high levels of endogenous OPN. This may be due to some combination of OPN effect on cell motility, as well as on properties related to the ability to breakdown and pass through a basement membrane barrier. Studies to examine the expression of various secreted proteases and their inhibitors are just underway, for both cells (21PT, 21NT, 21MT-1, and MDA-MB-435) induced by exogenous (hr)OPN, and the transfected (21PT and 21NT) cells expressing high levels of endogenous OPN. This work should help to identify genes related to invasiveness which may be coordinately regulated by OPN. Although results of *in vivo* assays for malignancy of both control and OPN-transfected cell lines were not yet available at the time of this report, the first series of both tail vein and mammary fat pad injections has been performed, such that we should have an indication of the manner in which the alterations in *in vitro* properties translate into behavior in *in vivo* assays by the time of the next annual report.

Interestingly, the pattern that appears to be forming in this work is that breast epithelial cells actually are capable of responding to OPN even from early stages of tumor progression (transformed, non-tumorigenic [i.e. 21PT]). At this early stage, the cells are already capable of responding to either exogenous or endogenous OPN by increased directed cell movement, and even by increased invasiveness through basement membrane in response to OPN. The difference between cells at various stages of progression may thus lie more in their **relative** ability to bind OPN (reflected in their degree of adhesiveness), and perhaps also in their relative ability to synthesize their own OPN (i.e. degree of autonomy for OPN expression). As stated above, this concept fits well with our finding in lymph node negative breast cancer patients that it is the

amount of OPN protein present in the tumor cells themselves (as opposed to within tumor infiltrating inflammatory cells), that predicts survival (manuscript submitted, See Appendix 1).

Other work from our group, published over the course of this year, suggesting a role for OPN in the malignancy of human breast cancer is included in Appendix 2 and 3. The latter of these (Appendix 3) was submitted just prior to the onset of the U.S. Army B.C.R.P. Career Development award. The Career Development award allows for followup studies related to this work. Abstracts involving the work presented in this report have been submitted for consideration for the U.S. Army Breast Cancer Research Program "Era of Hope" meeting October 31 - November 4, 1997, and the San Antonio Breast Cancer Symposium, December 3-6, 1997. Manuscripts are presently in preparation based on the work described here related to the effects of OPN on cell adhesion, migration, and invasiveness, and on the integrin-dependence of the cell migration effects and synergistic relationships of OPN induction with EGF and HGF.

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FIGURE 1

Northern analysis of OPN mRNA expression by 21T series human mammary epithelial cell lines vs. MDA-MB-435 (highly metastatic human breast carcinoma) cells. Total RNA (10 μg/lane) was separated, blotted, and probed as described in Methods. The OPN mRNA message size was 1.8 kb. Equivalent RNA loading and integrity were verified by assessment of 18s rRNA bands (not shown).

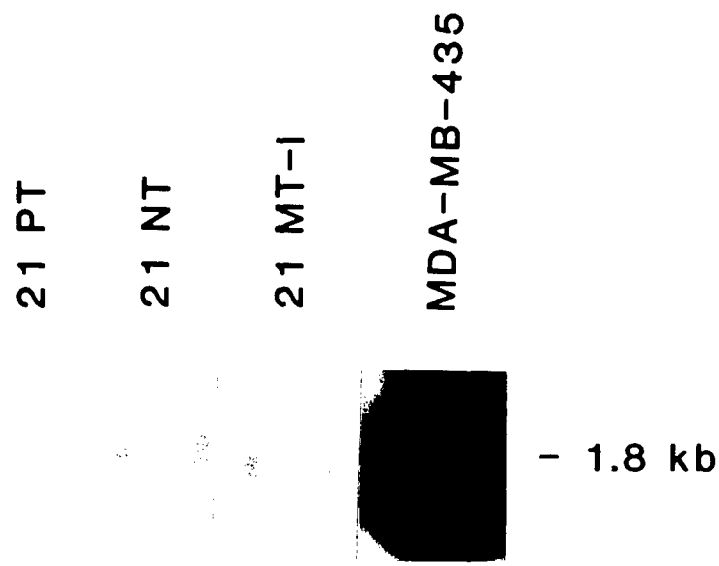

21 PT
21 NT
21 MT-1
MDA-MB-435
- 1.8 kb

FIGURE 2

Western blot analysis of secreted OPN of 21T series cells vs. MDA-MB-435 cells. Protein was fractionated in 12% SDS-PAGE and detected with mAb53 as described in Methods. Serum-free culture supernatants were concentrated by microfiltration prior to fractionation in SDS-PAGE. The equivalent of 8-10 μg of total secreted protein was loaded after correction for cell equivalents based on cell counts at the time of collection of conditioned media (see Methods). Molecular mass markers used were biotinylated protein standards (BioRad).

Figure 2

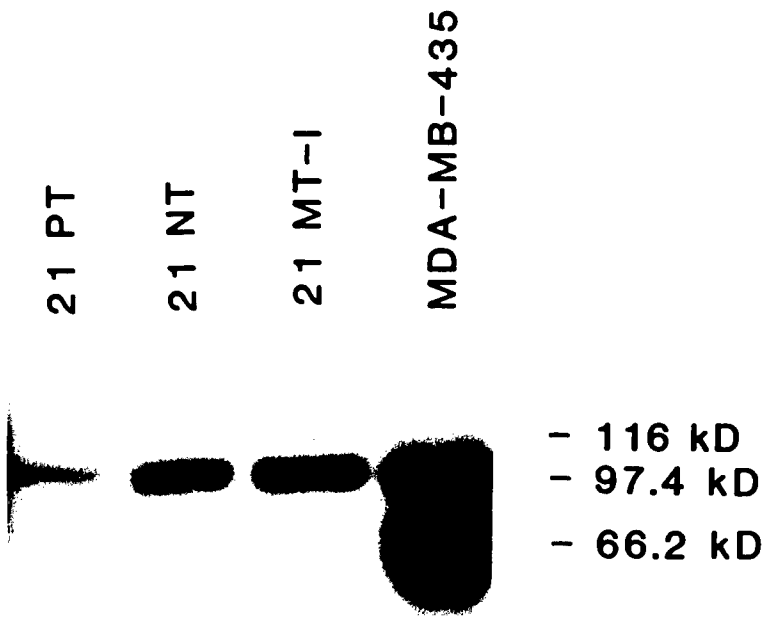


FIGURE 3

ELISA assay of secreted OPN protein in conditioned media of 21PT and 21 NT cells transfected with either the vector control DNA alone, or with the OPN-containing expression vector as described in Methods. Unconcentrated conditioned media were assayed using an antigen capture assay as described in Methods, and are expressed as values relative to background (BSA-only control). Cell lines are as follows: PT/Ci and NT/Ci: Pooled transfectant populations of 21PT and 21NT cells respectively, transfected with the control vector; PT/OPaiiPool: Pooled transfectant population of 21PT cells, transfected with the OPN-containing expression vector; PT/OPaiiC12: Clone 12 of tranfectant population PT/OPaii; NT/OPbiPool: Pooled transfectant population of 21NT cells, transfected with the OPN-containing expression vector; NT/OPaiiC4: Clone 4 of transfectant population NT/OPaii.

FIGURE 3

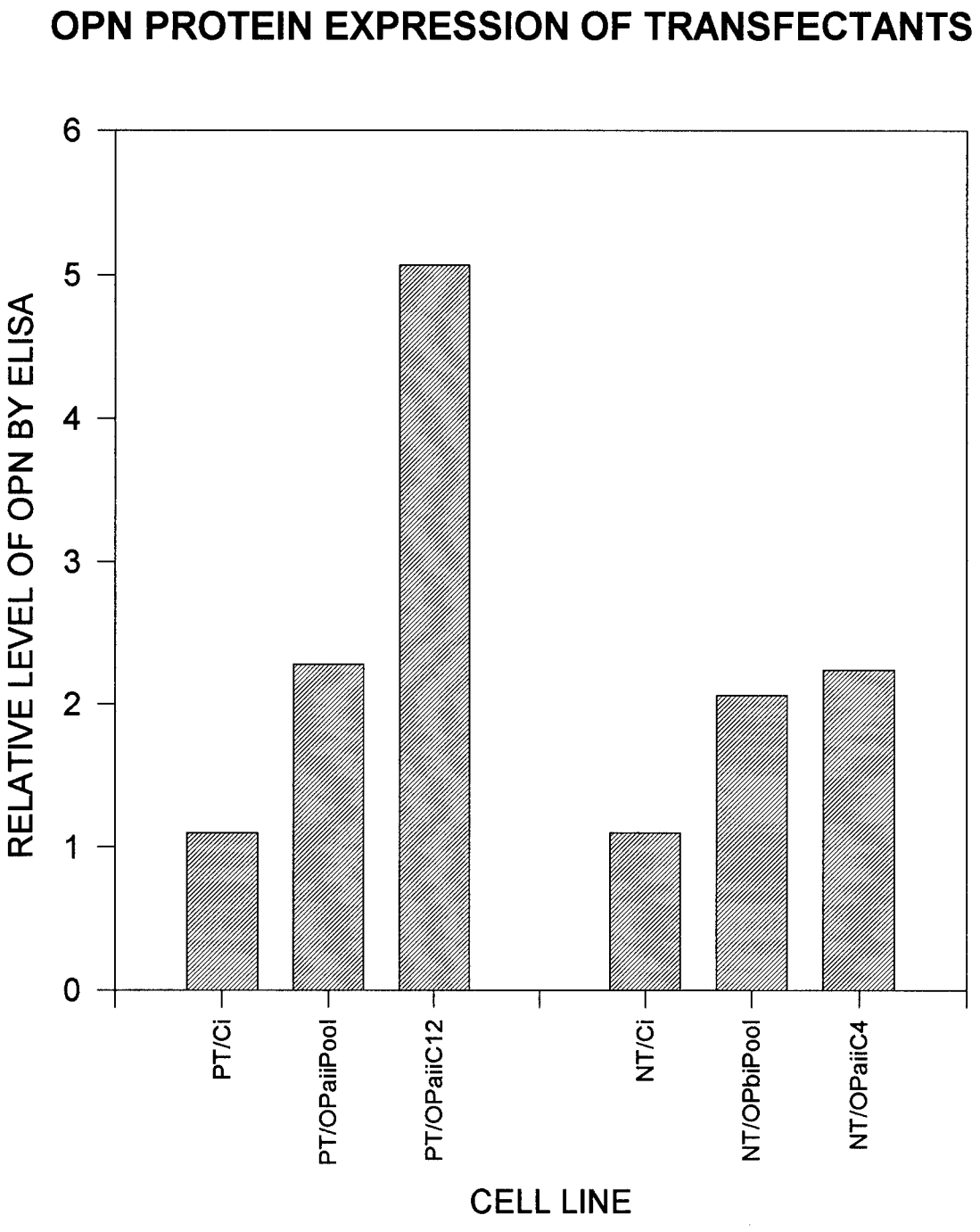

OPN PROTEIN EXPRESSION OF TRANSFECTANTS
RELATIVE LEVEL OF OPN BY ELISA
0
1
2
3
4
5
6
PT/Ci
PT/OPaiiPool
PT/OPaiiC12
NT/Ci
NT/OPbiPool
NT/OPaiiC4
CELL LINE

FIGURE 4

Northern analysis of OPN mRNA expression by 21PT and 21NT cells transfected with vector control DNA (PT/Ci, NT/Ci) vs. the OPN-containing expression vector (PT/OPaiiPool, PT/OPaiiC12, NT/OPbiPool, NT/OPaiiC4) as described in Methods, compared with untransfected MDA-MB-435 cells. PT/OPaiiPool and NT/OPbiPool are pooled populations of transfectants, whereas PT/OPaiiC12 and NT/OPaiiC4 represent cloned populations. Total RNA (10 μg/lane) was separated, blotted, and probed as described in Methods. The OPN mRNA message size was 1.8 kb. Equivalent RNA loading and integrity were verified by assessment of 18s rRNA bands (not shown).

FIGURE 4

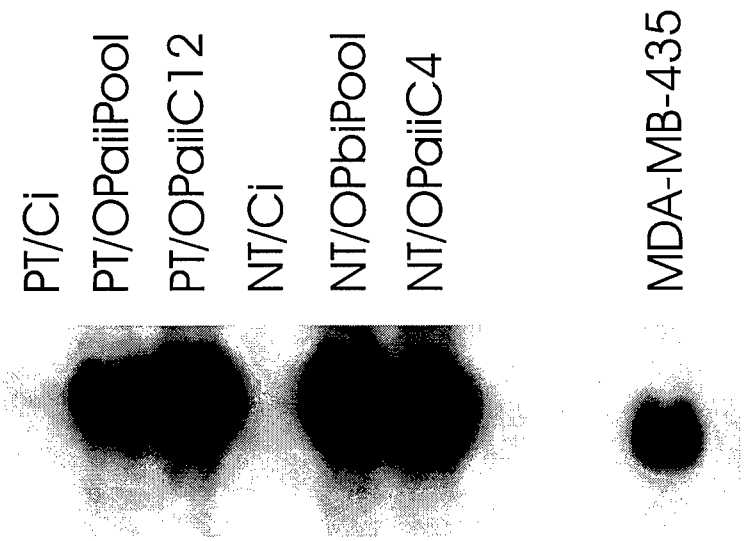

PT/Ci
PT/OPaiiPool
PT/OPaiiC12
NT/Ci
NT/OPbiPool
NT/OPaiiC4
MDA-MB-435

FIGURE 5

Serum dependence of the 21T series parental cell lines (21PT, 21NT, 21MT-1). Cells were plated in triplicate at 5 X 10^4 cells/plate on 60mm plates under one of three conditions: α-medium with hydrocortisone (2.8 μM) and EGF (12.5 ng/ml) (αHE) with 10% FBS [HE 10F]; αHE with 0.5% FBS [HE 0.5F]; or OPTI MEM with 5% FBS plus hydrocortisone and EGF [OPTI 5F]. Triplicate plates per condition were trypsinized and counted on the specified days.

FIGURE 5

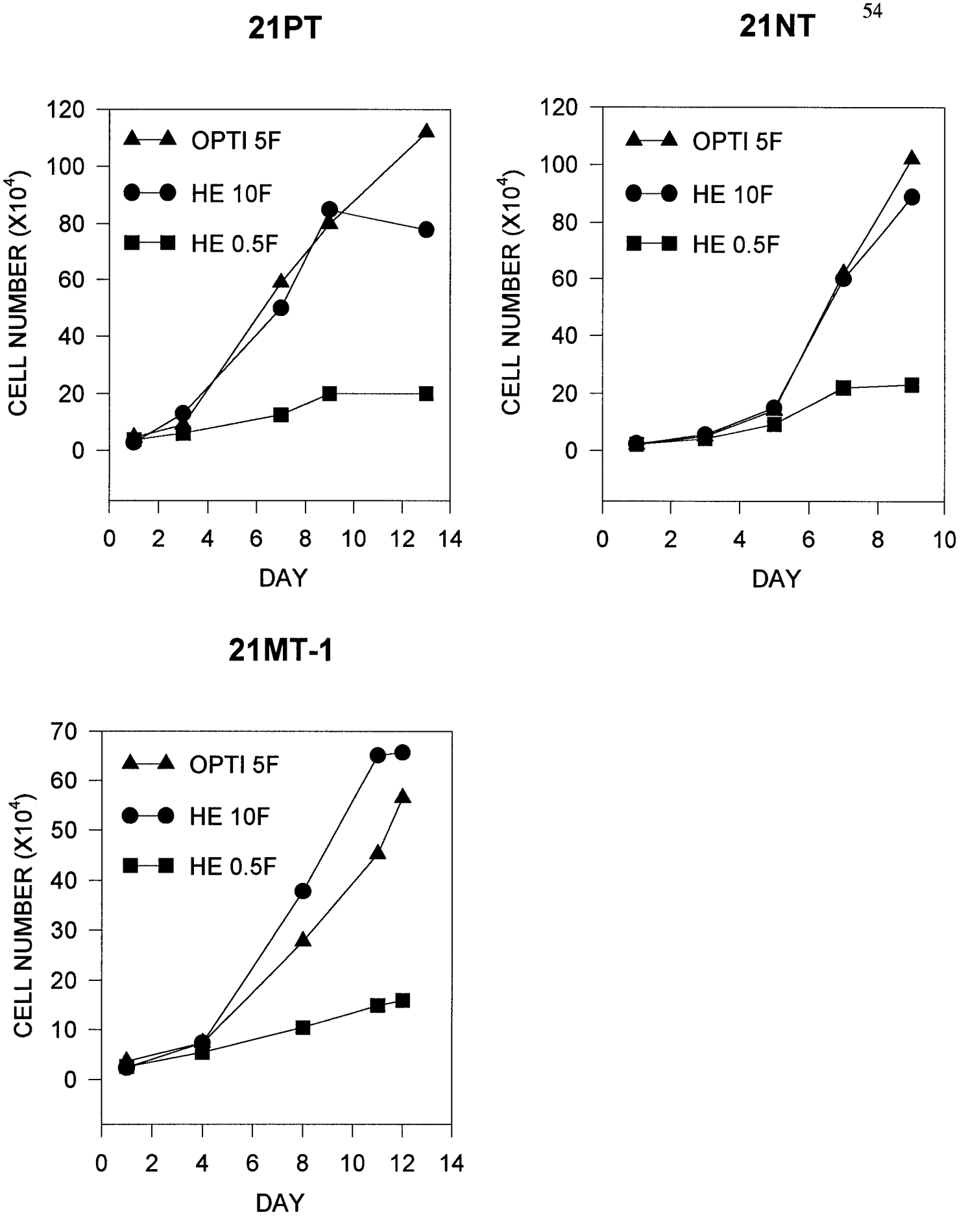

21PT
21NT
21MT-1
OPTI 5F
HE 10F
HE 0.5F
CELL NUMBER (X10⁴)
DAY
0
20
40
60
80
100
120
2
4
6
8
10
12
14
30
50
70

FIGURE 6

Cell growth in low serum with or without addition of exogenous hrOPN (50 μg/ml). Cells were plated on 24 well plates at 5 X 10^3 cells/well in αHE with 0.5% FBS. Duplicate wells were seeded per time point per cell line, either in the presence or absence of 50 μg/ml hrOPN. Cells were allowed to grow for the specified time period, trypsinized and counted. Cell lines: Parental 21PT, 21NT, 21MT-1 and MDA-MB-435 cells.

FIGURE 6

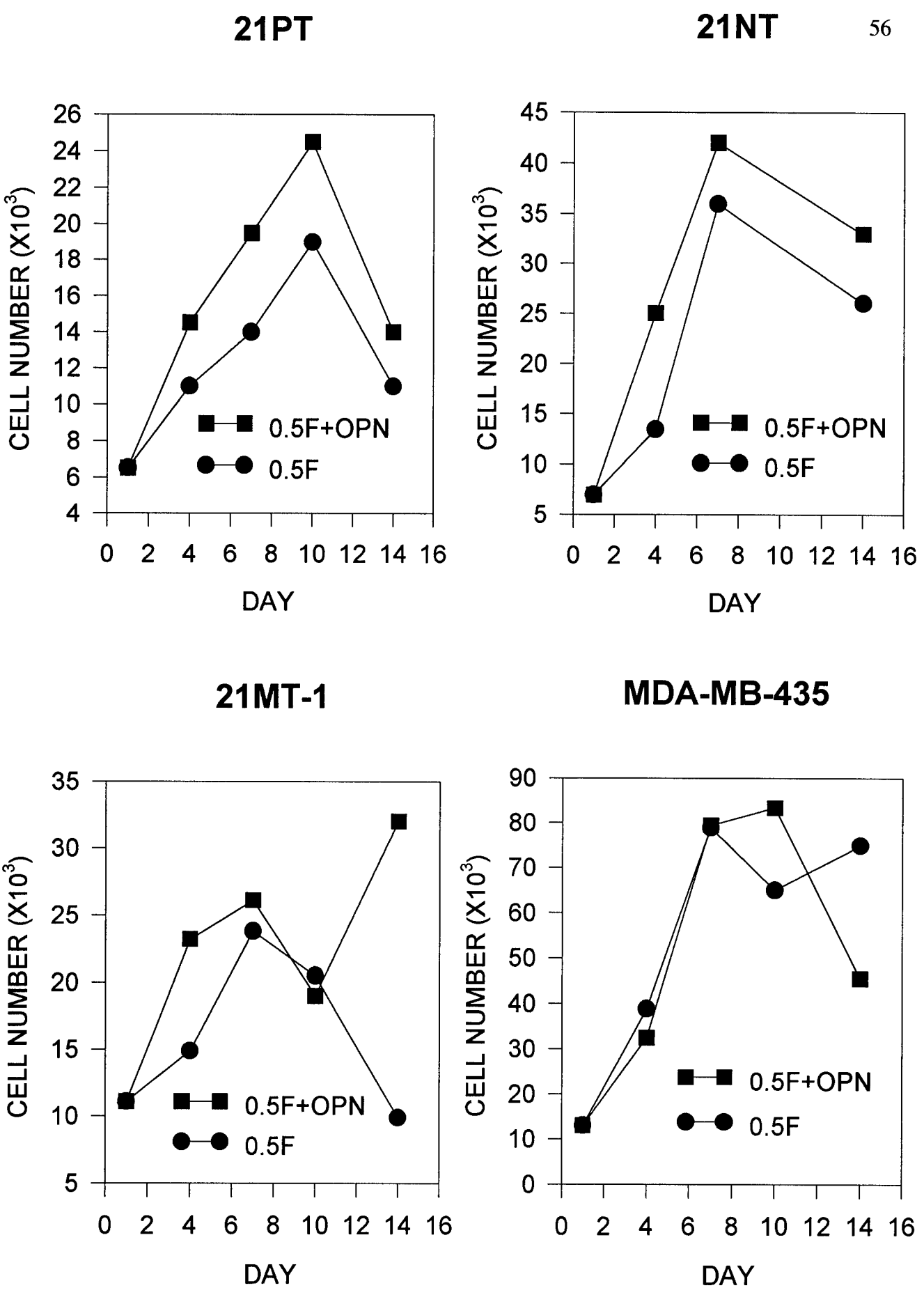


FIGURE 7

Adhesion of cells to coated surfaces. 21PT (PT), 21NT (NT), 21MT-1 (MT), and MDA-MB-435 (MDA) cells were added to 96-well plates that had been coated with hrOPN (OP), RGD deletion mutant hrOPN (mutOP), or BSA only (0), all at 10 μg/ml. Cells were allowed to adhere for 2 hours, and plates were washed, fixed, stained and counted as described in Methods. Attached cells per high power field were counted (field area 0.13 mm^2). The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 7

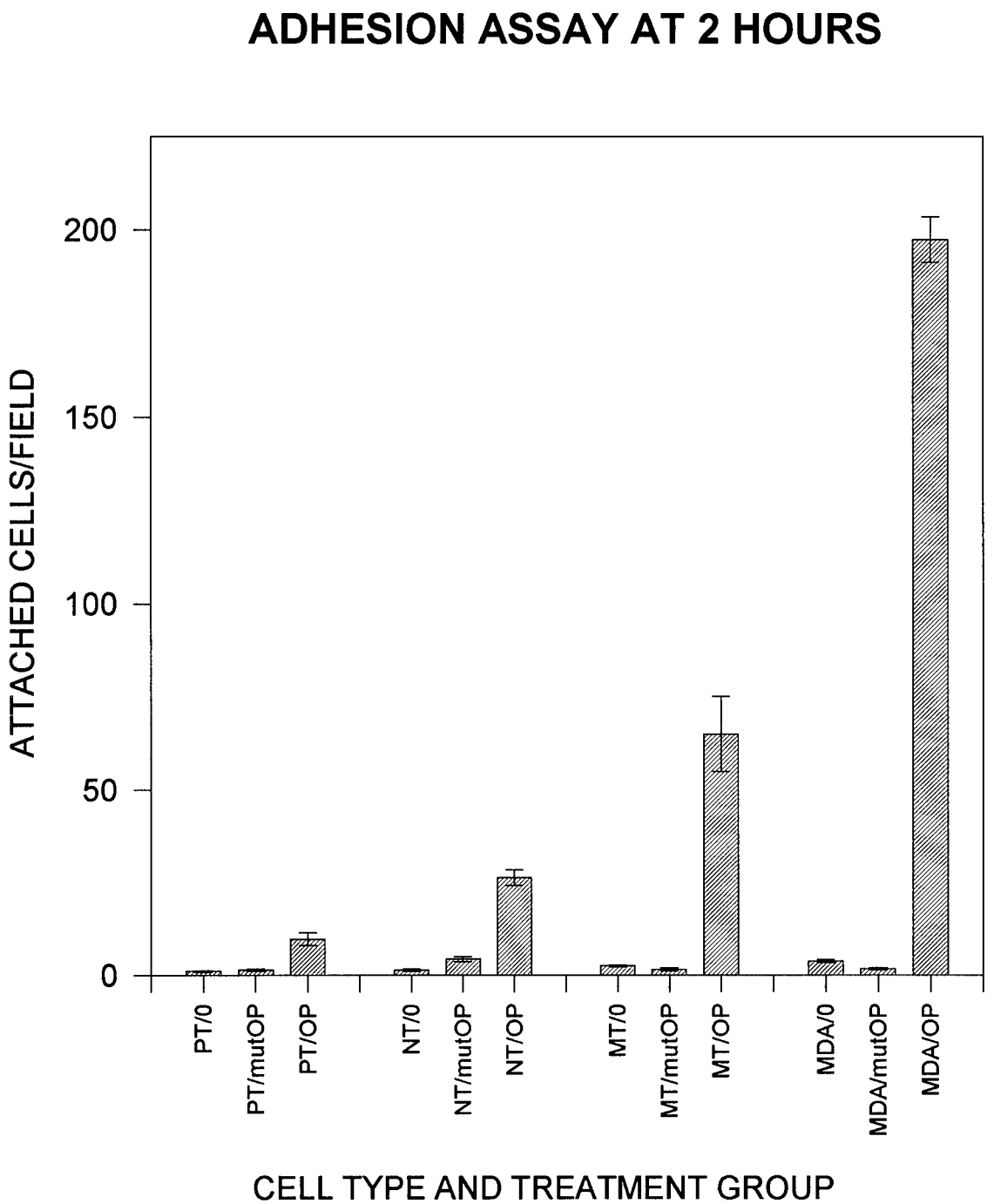

ADHESION ASSAY AT 2 HOURS
ATTACHED CELLS/FIELD
200
150
100
50
0
PT/0
PT/mutOP
PT/OP
NT/0
NT/mutOP
NT/OP
MT/0
MT/mutOP
MT/OP
MDA/0
MDA/mutOP
MDA/OP
CELL TYPE AND TREATMENT GROUP

FIGURE 8

Cell migration response to hrOPN (50 μg/ml) (OP) vs. BSA (0.1%) (0) in lower chamber of 8 μm pore transwells. Cells (21PT [PT], 21NT [NT], 21MT-1 [MT], or MDA-MB-435 [MDA]) were seeded at 5 X 10^4 cells/well in the upper chamber, and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 8

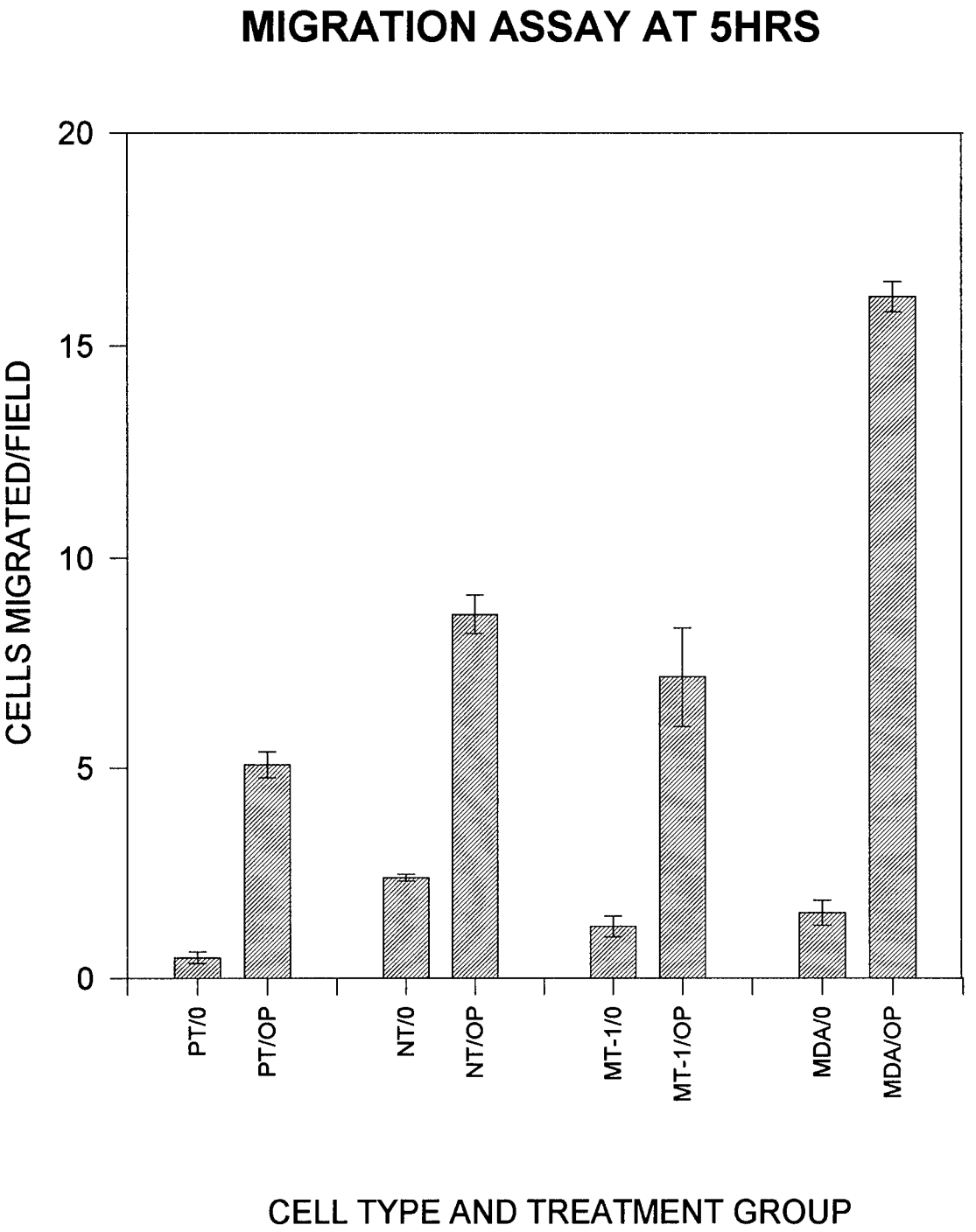

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
20
15
10
5
0
PT/0
PT/OP
NT/0
NT/OP
MT-1/0
MT-1/OP
MDA/0
MDA/OP
CELL TYPE AND TREATMENT GROUP

FIGURE 9

Cell migration of 21PT (PT) and 21NT (NT) cells in response to different concentrations of hrOPN (50 μg/ml vs. 100 μg/ml) (OP50; OP100), as compared to 0.1% BSA alone (0) in the lower chamber of 8 μm pore transwells. Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 9

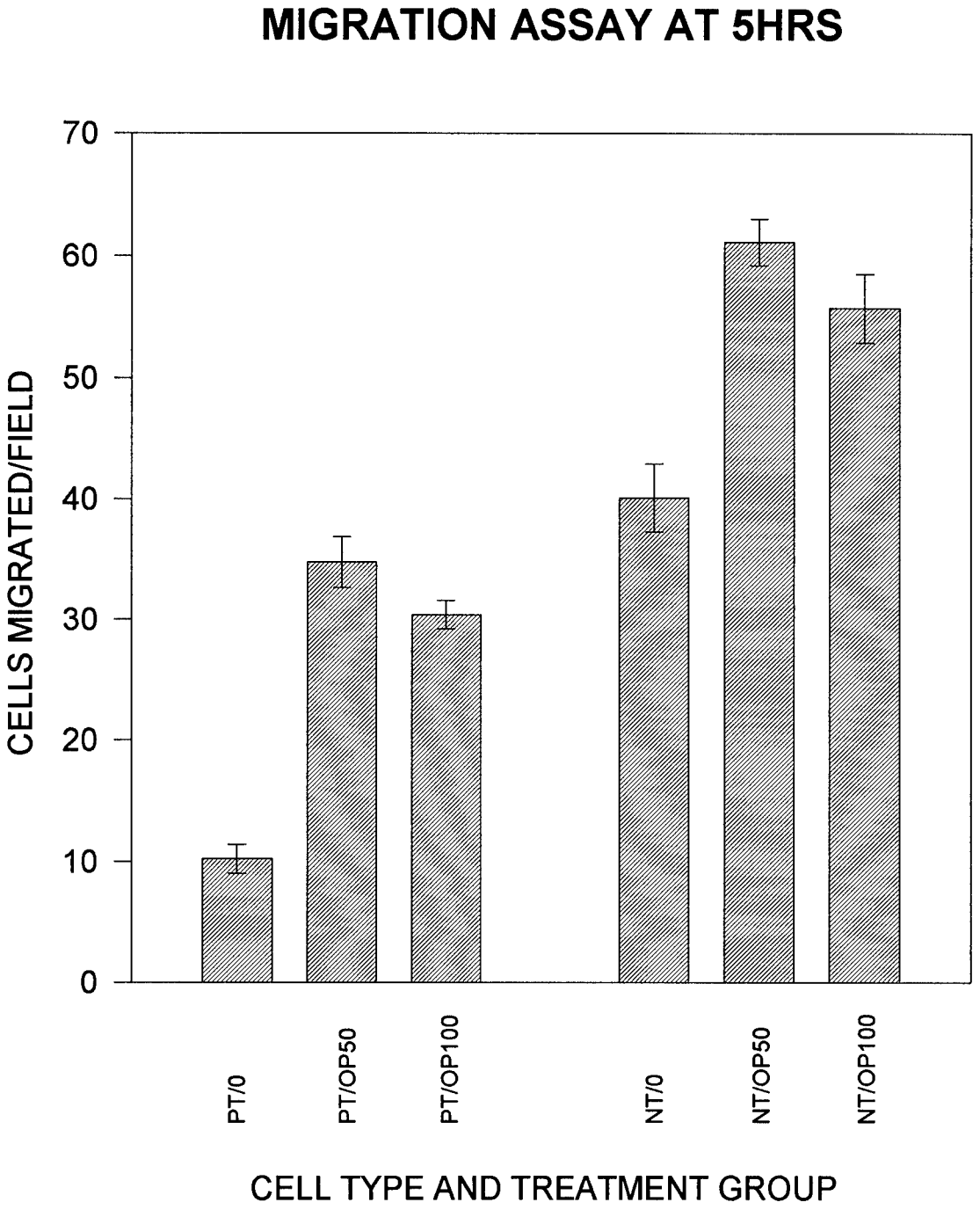

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
70
60
50
40
30
20
10
0
PT/0
PT/OP50
PT/OP100
NT/0
NT/OP50
NT/OP100
CELL TYPE AND TREATMENT GROUP

FIGURE 10

"Checkerboard" analysis of cell migration of 21PT (PT) and 21NT (NT) cells to 50 μg/ml hrOPN in lower chambers of 8 μm pore transwells (-/+), both chambers (+/+), upper chamber (+/-), or neither chamber (-/-). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Multiple pairwise comparisons indicated that: for 21PT cells, only OPN present in the lower chamber was significantly different from the other conditions ($p < 0.05$); for 21NT cells, OPN present in the lower chamber was significantly different than all other conditions ($p < 0.05$), but also OPN present in the upper chamber only was significantly different from no OPN ($p < 0.05$).

FIGURE 10

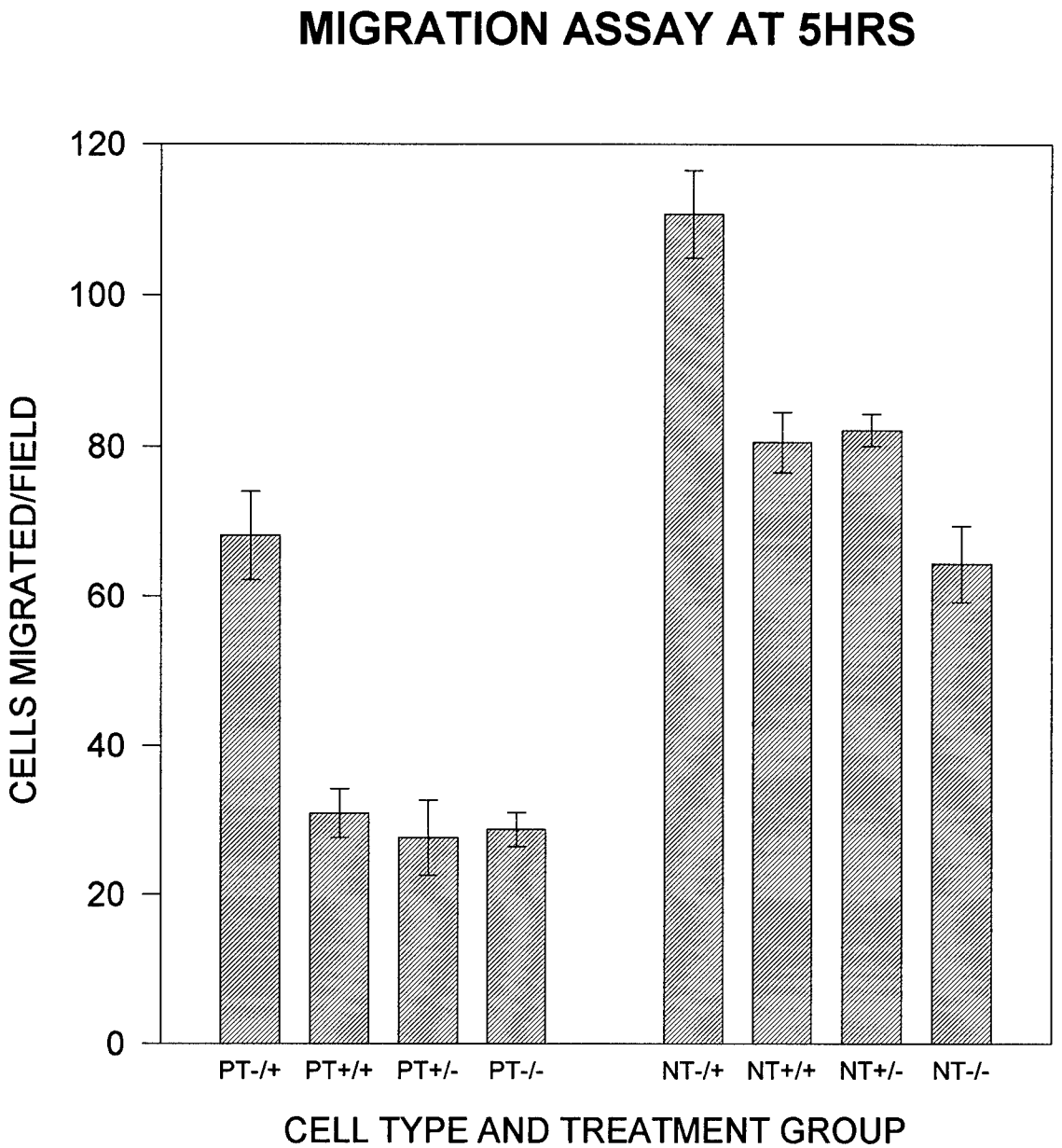

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
120
100
80
60
40
20
0
PT-/+
PT+/+
PT+/-
PT-/-
NT-/+
NT+/+
NT+/-
NT-/-
CELL TYPE AND TREATMENT GROUP

FIGURE 11

Anti-OPN antibody (mAb53) blocking of hrOPN-induced cell migration. Medium in the lower chamber of 8 μm pore transwells contained either: 0.1% BSA only (0); 50 μg/ml hrOPN (OP); or 50 μg/ml hrOPN plus 20 μg/ml anti-OPN antibody (OPAb). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Addition of anti-OPN antibody significantly reduced the OPN-induced migration of both 21PT (PT) and 21NT (NT) cells ($p < 0.05$ for both, one-way ANOVA). 21NT cells showed a level of migration significantly below baseline in the presence of anti-OPN antibody ($p < 0.05$, one-way ANOVA).

FIGURE 11

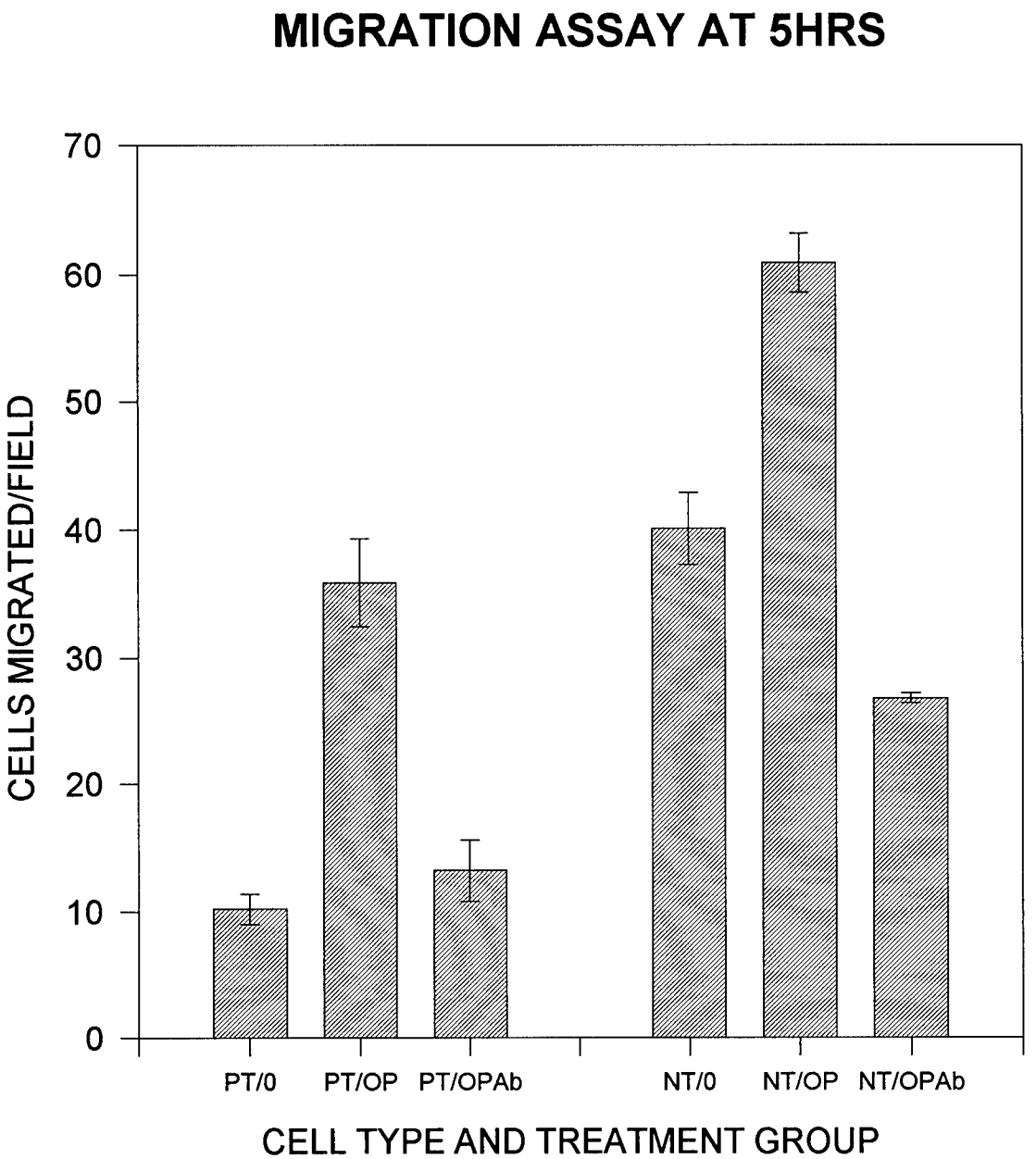

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
70
60
50
40
30
20
10
0
PT/0
PT/OP
PT/OPAb
NT/0
NT/OP
NT/OPAb
CELL TYPE AND TREATMENT GROUP

FIGURE 12

RGD-dependence of cell migration response to hrOPN. Medium in the lower chamber of 8 μm pore transwells contained either: 0.1% BSA only (0); 50 μg/ml hrOPN (OP); 50 μg/ml hrOPN plus 100 μM GRGDS peptide (OP+RGDp); or RGD deletion mutant OPN (OPmut). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Addition of GRGDS peptide significantly reduced the OPN-induced migration of both 21PT (PT) and 21NT (NT) cells ($p < 0.05$ for both, one-way ANOVA) to levels which were not significantly different from baseline. Use of the RGD deletion mutant hrOPN resulted in no significant induction of cell motility in either 21PT or 21NT.

FIGURE 12

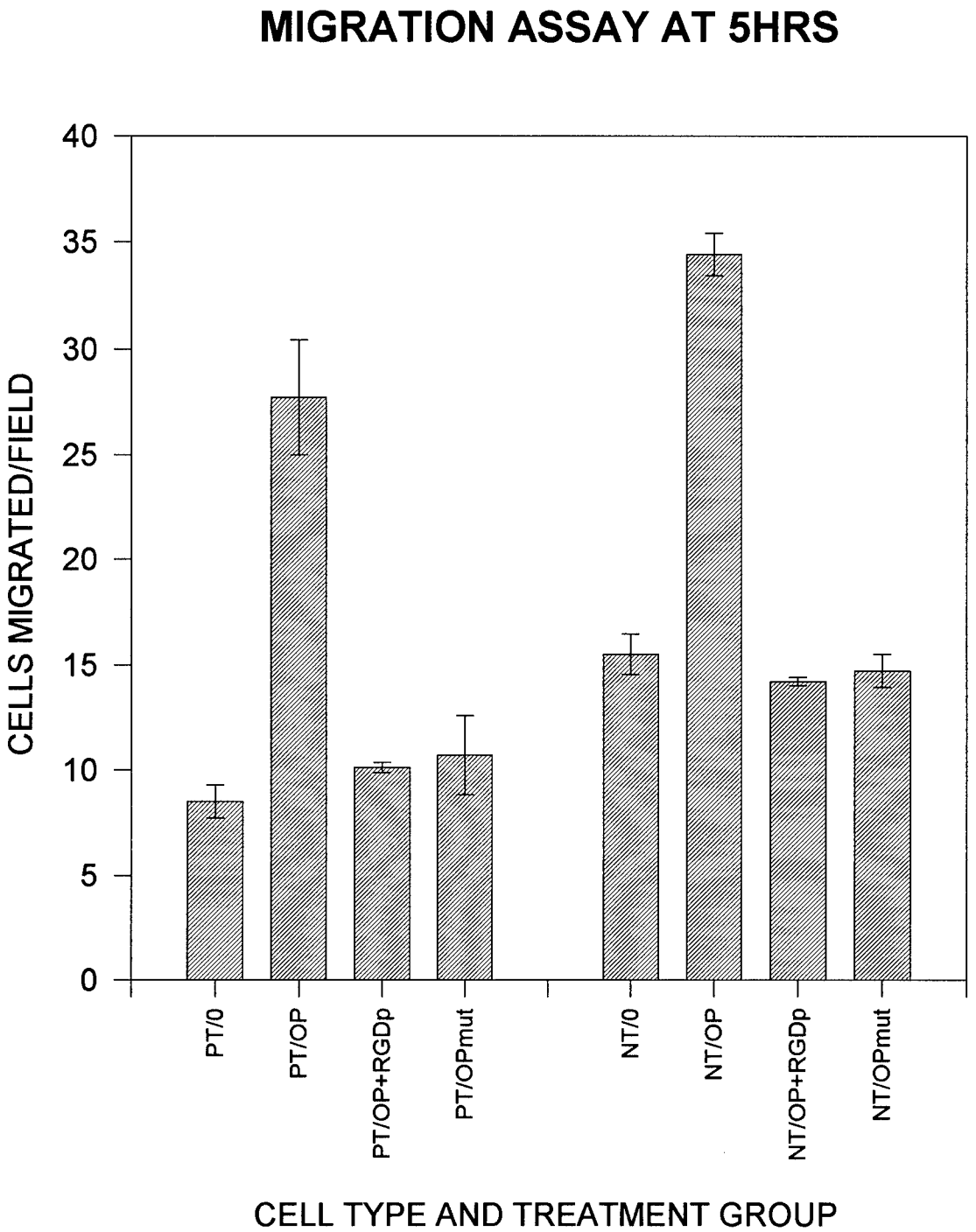

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
0
5
10
15
20
25
30
35
40
PT/0
PT/OP
PT/OP+RGDp
PT/OPmut
NT/0
NT/OP
NT/OP+RGDp
NT/OPmut
CELL TYPE AND TREATMENT GROUP

FIGURE 13

Cell migration of 21PT and 21NT cells in response to EGF and hrOPN, alone and in combination. Contents of the lower chamber consisted of either: medium without EGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 12.5 ng/ml EGF (EGF); or medium with 50 μg/ml hrOPN and 12.5 ng/ml EGF (OP+EGF). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Both 21PT (PT) and 21NT (NT) cells showed significantly increased migration in response to either hrOPN or EGF alone (all show $p < 0.05$ by one-way ANOVA). No significant difference was apparent for either cell line between hrOPN effect alone vs. EGF effect alone. For both 21PT and 21NT cells, the effect of hrOPN in combination with EGF was significantly greater than either agent alone ($p < 0.05$ by one-way ANOVA in all pairwise comparisons), and the combined effect was more than additive (i.e. synergistic).

FIGURE 13

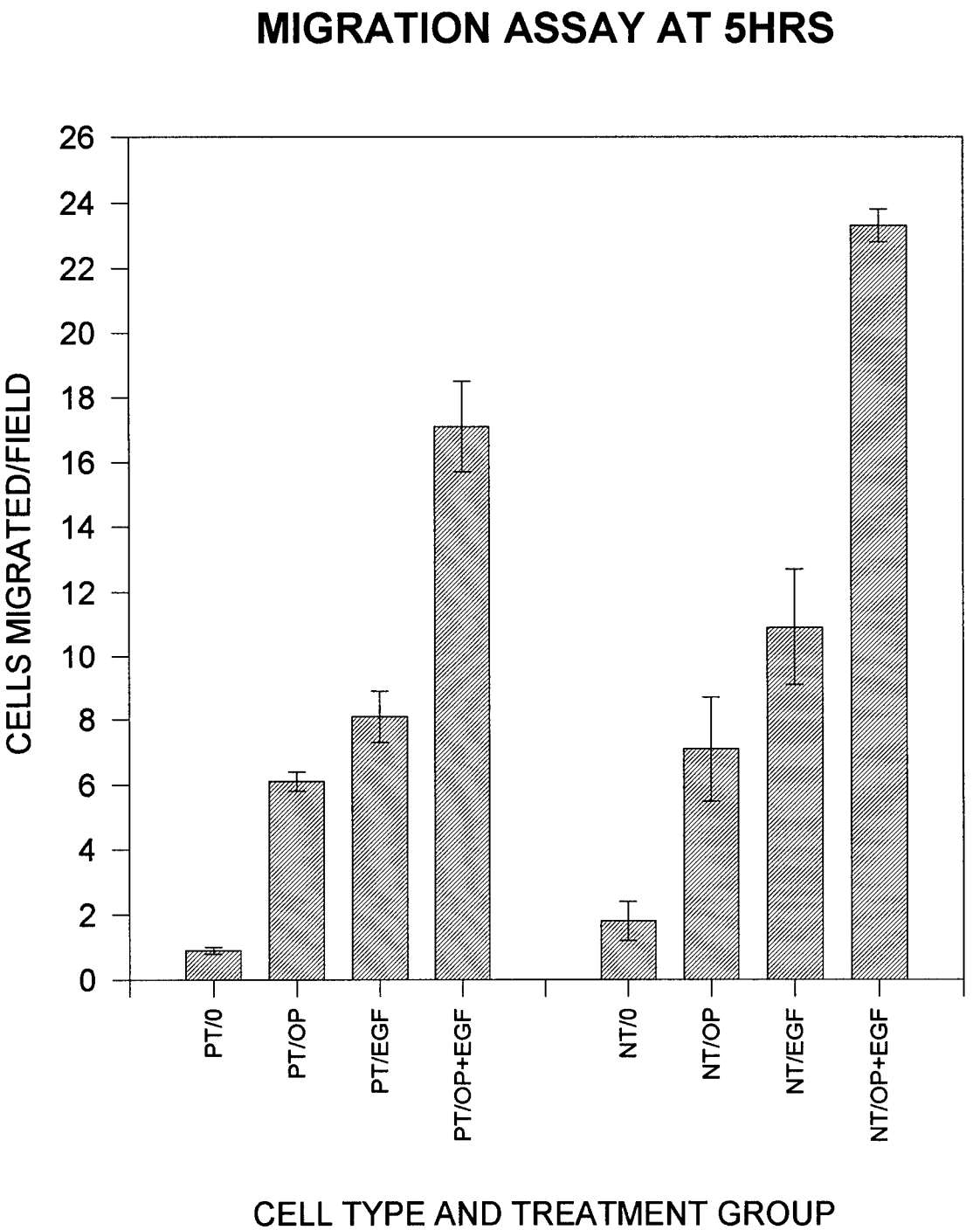

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
0
2
4
6
8
10
12
14
16
18
20
22
24
26
PT/0
PT/OP
PT/EGF
PT/OP+EGF
NT/0
NT/OP
NT/EGF
NT/OP+EGF
CELL TYPE AND TREATMENT GROUP

FIGURE 14

Cell migration of 21PT (PT) and 21NT (NT) cells in response to HGF and hrOPN, alone and in combination. Contents of the lower chamber consisted of either: medium (αH, no EGF) without HGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 10 ng/ml HGF (HGF); or medium with 50 μg/ml hrOPN and 10 ng/ml HGF (OP+HGF). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Multiple pairwise comparisons showed all four treatment conditions for both 21PT and 21NT to be significantly different from each other (all show $p < 0.05$ by one-way ANOVA). For both 21PT and 21NT cells, the effect of hrOPN in combination with HGF was more than additive (i.e. synergistic).

FIGURE 14

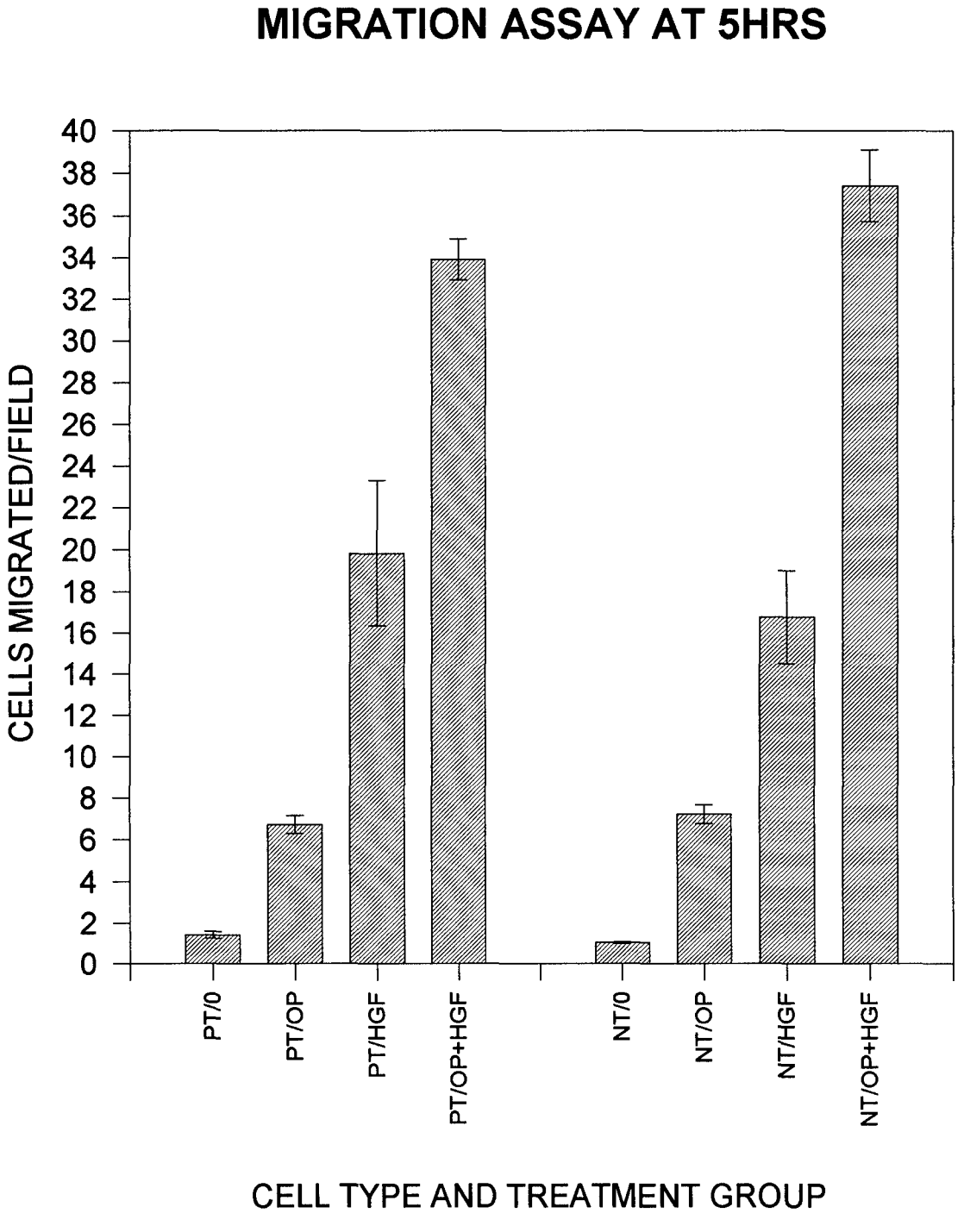

MIGRATION ASSAY AT 5HRS
CELLS MIGRATED/FIELD
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
PT/0
PT/OP
PT/HGF
PT/OP+HGF
NT/0
NT/OP
NT/HGF
NT/OP+HGF
CELL TYPE AND TREATMENT GROUP

FIGURE 15

In vitro chemoinvasiveness of 21PT (PT), 21NT (NT), 21MT-1 (MT-1) and MDA-MB-435 (MDA) cells in response to 100 μg/ml hrOPN (OP) in the lower chamber of 8 μm pore transwells, vs. 0.1% BSA only (0). Filters of each transwell were coated with 35 μg Matrigel as described in Methods. Cells were seeded at 7.5 X 10^4 cells/well in the upper chamber and incubated for 72 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted. The *bar graphs* represent the mean of counts from each of three separate wells and the error bars represent the standard error of the mean. All four cell lines show significantly increased invasion in response to hrOPN ($p < 0.05$ by student's *t*-test for all).

FIGURE 15

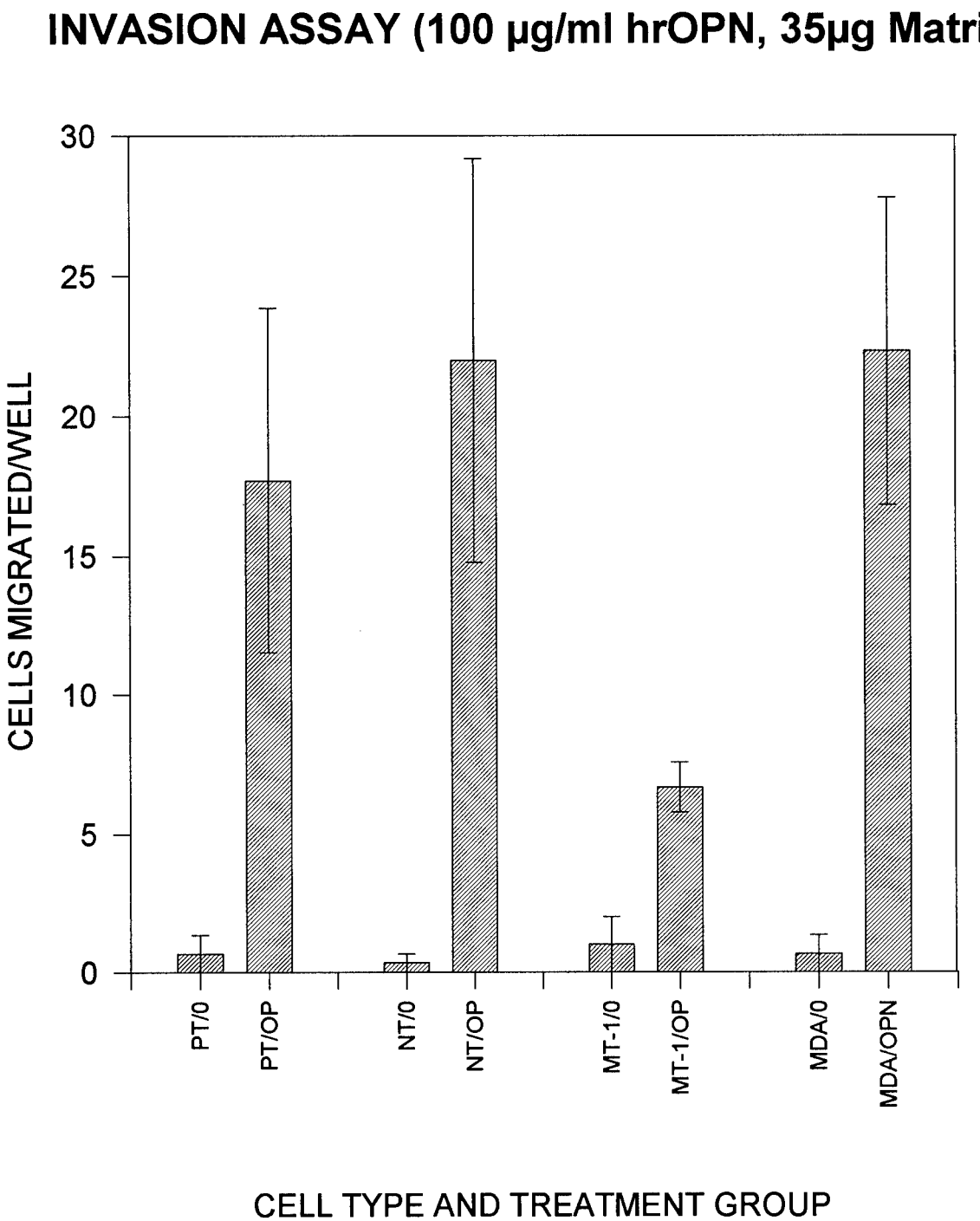
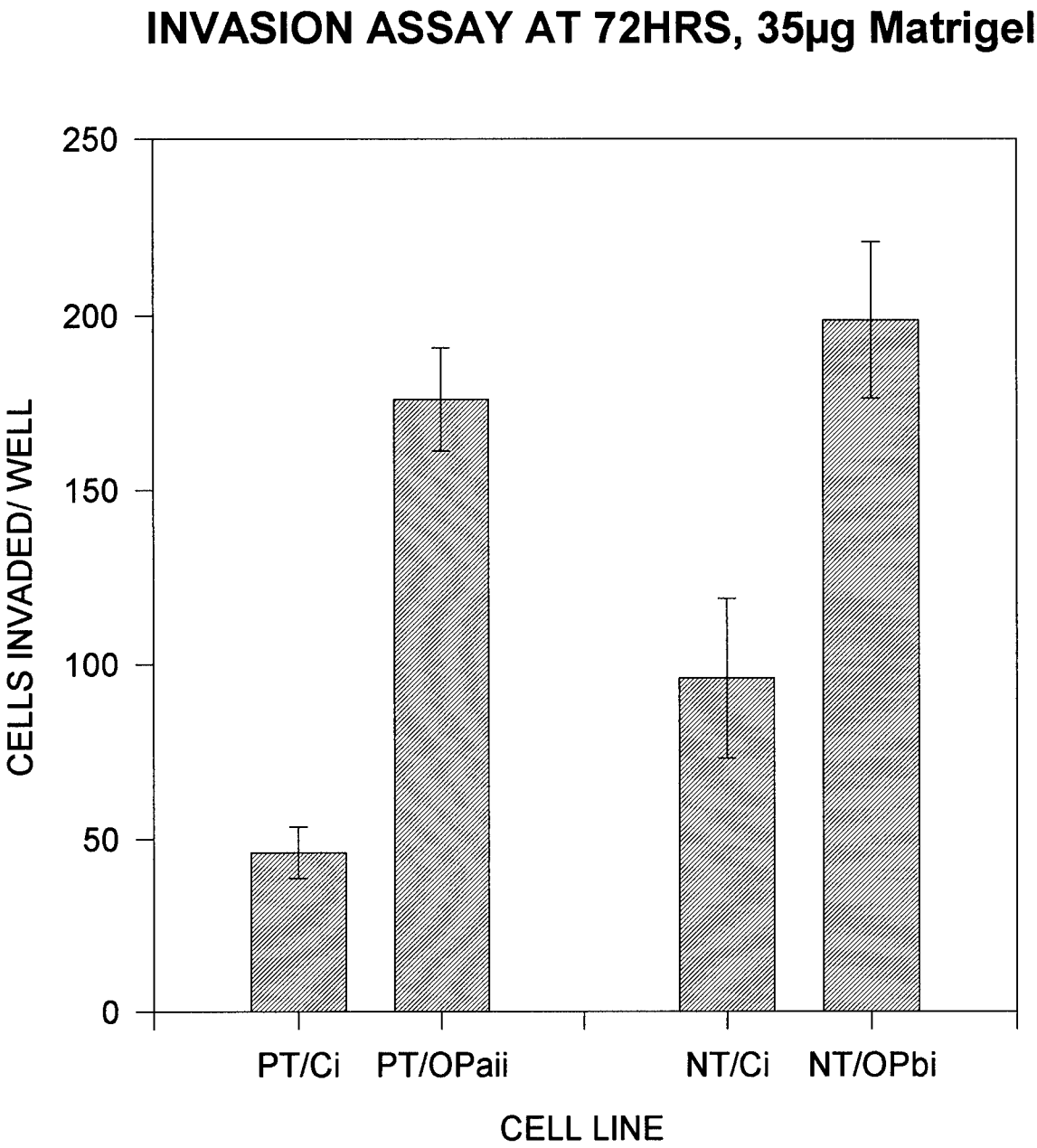

INVASION ASSAY (100 µg/ml hrOPN, 35µg Matrigel)
CELLS MIGRATED/WELL
30
25
20
15
10
5
0
PT/0
PT/OP
NT/0
NT/OP
MT-1/0
MT-1/OP
MDA/0
MDA/OPN
CELL TYPE AND TREATMENT GROUP

FIGURE 16

In vitro chemoinvasiveness of transfectant cell lines in the transwell assay. Vector-only transfected controls of 21PT (PT/Ci) and 21NT (NT/Ci) cells are compared with the OPN-transfected pool of 21PT (PT/OPaii) and 21NT (NT/OPbi) cells expressing the highest levels of OPN. Invasion is in response to 10 μg/ml fibronectin in the lower chamber, through 8 μm pore filters precoated with 35 μg Matrigel, as described in Methods. Cells were seeded at 7.5 X 10^4 cells/well in the upper chamber and incubated for 72 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted. The *bar graphs* represent the mean of counts from each of three separate wells and the error bars represent the standard error of the mean. OPN-transfected 21PT (PT/OPaii) and 21NT (NT/OPbi) both showed significantly increased invasiveness over the vector-only transfected controls (PT/Ci, NT/Ci) ($p=0.0014$ and $p=0.0327$ respectively by the student's *t*-test).

FIGURE 16


INVASION ASSAY AT 72HRS, 35µg Matrigel
250
200
150
100
50
0
CELLS INVADED/ WELL
PT/Ci
PT/OPaii
NT/Ci
NT/OPbi
CELL LINE

TUMOR CELL OSTEOPONTIN (OPN) IS ASSOCIATED WITH A MORE AGGRESSIVE CLINICAL COURSE IN PATIENTS WITH LYMPH NODE NEGATIVE BREAST CANCER

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Running Title: Osteopontin in LNN Breast Cancer

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Number of text pages: 19 Tables: 1 Figures: 3

ABSTRACT

The aim of this study was to examine the cellular distribution of osteopontin (OPN) protein (by immunohistochemical [IHC] analysis) and mRNA (by in situ hybridization [ISH]) in the primary tumors of lymph node negative (LNN) breast cancer patients, and to determine whether level of immunodetectable OPN may be of prognostic value in this setting. We examined OPN levels in tumors from 154 patients with lymph node negative breast cancer, who were followed for a median of 7 years (range 1.7 to 16.3). IHC staining for OPN was seen in tumor infiltrating macrophages and lymphocytes in 70% of these tumors, and in the carcinoma cells themselves in 26%. Tumor cell IHC staining intensity was assessed using a semiquantitative scoring system. Univariate analysis showed tumor cell OPN positivity (score >4) to be significantly associated with decreased disease free survival (DFS) and overall survival (OS). In the multivariate model, which included patient age, menopausal status, tumor size, grade, hormone receptor status, and p53 immunopositivity, tumor cell OPN positivity (score >4) remained a significant predictor of decreased OS ($p=0.0138$, $RR=2.971$) but not DFS ($p=0.3217$, $RR=1.634$). *In situ* hybridization was performed to determine cellular distribution of OPN mRNA expression in sections from selected tumors. OPN mRNA was detected in tumor infiltrating macrophages and lymphocytes, as well as in groups of invading tumor cells. OPN protein in tumor cells thus can be produced by tumor cells themselves, and could also be taken up from the environment (secreted by inflammatory cells or other tumor cells). Regardless of the cellular source of OPN, this pilot study shows significant independent prognostic value for IHC determination of *tumor cell* OPN in lymph node negative breast cancer patients, and suggests the need for further definitive study to substantiate this finding.

INTRODUCTION

Osteopontin (OPN) is a secreted, adhesive glycophosphoprotein which has been implicated in both normal (eg. bone development, immune system regulation) and pathologic (eg. transformation, kidney stone formation) processes. OPN has been found to be expressed by a number of different cell types including osteoblasts, arterial smooth muscle cells, leukocytes (particularly activated macrophages and T-cells), various types of epithelial cells and transformed cells of different lineages (1 [review]).

In normal breast tissue, OPN is expressed by secretory phase ductal epithelium, occasionally by non-lactating breast epithelial cells, and is seen to be localized on the apical (luminal) aspect of the cells (2). Elevated plasma levels of OPN have been reported in patients with metastatic carcinoma, including metastatic breast cancer (3-6). We have recently reported an association between high plasma level of OPN, increased tumor burden, and decreased survival in patients with metastatic breast cancer (5,6). OPN has also been detected in the primary tumors of patients with breast cancer (7-9), where evidence has suggested expression by tumor infiltrating macrophages. To this point, there has been no association established between levels of expression of tumor OPN and prognosis in patients with breast cancer.

Although OPN protein has been detected by immunohistochemistry (IHC) in the tumor cells themselves (7,9), a lack of *in situ* evidence for RNA expression by the cancer cells has led Brown et al. (7) to suggest that OPN secreted by macrophages may bind to and be taken up by the tumor cells. However, there is experimental evidence that cultured mammary carcinoma cells (eg. D2HAN series [10,11]; MDA-MB-435 cells [12]) not only may produce OPN, but that at least some (MDA-MB-435 cells) show RGD-dependent adhesion to and migration towards

OPN in culture (12-15).

We have here undertaken a study to examine the expression of OPN in the primary tumors of a group of 154 lymph node negative breast cancer patients, in order to determine if there is a relationship between OPN positivity and outcome, as well as to further examine the cellular distribution of OPN expression.

MATERIALS AND METHODS

Patients: One hundred and fifty four patients with lymph node negative (LNN) breast cancer were identified from the records at the London Regional Cancer Centre (London, Ontario, Canada). Data available included age, menopausal status, tumor size, biochemical ER and PR receptor status, p53 status, and definitive surgical treatment. Dates of recurrence or death were recorded as well as date of last follow-up for those who remained disease-free. All of the 154 patients showed either invasive or microinvasive mammary carcinoma.

OPN Immunohistochemistry: Formalin-fixed, paraffin-embedded tumor samples were assessed for OPN expression by an immunoperoxidase technique. Representative four micron sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 minutes. An antigen retrieval method was used on all slides prior to immunostaining. This involved heating tissue sections in citrate buffer (10mM, pH 6.0) in a microwave oven (600 watt) for 7 minutes. Immunostaining was performed using a streptavidin-biotin complex method (Zymed histostain kit-Dimension Laboratories, Toronto, ON). Non-specific staining was blocked by incubating slides with 5% normal goat serum. The

primary antibody used was monoclonal antibody mAb53, prepared against the recombinant GST-human OPN fusion protein (12). This antibody has been previously shown by Western analysis and ELISA assay to efficiently detect human OPN (12,16). The slides were incubated with primary antibody for one hour at room temperature. Slides were then rinsed with PBS, and a biotinylated secondary antibody was applied for 15 minutes, followed by a PBS rinse and treatment with the streptavidin-enzyme conjugate for 10 minutes. The chromogen used was aminoethyl carbazol (reddish-brown signal), and slides were counterstained with Mayer's hematoxylin. Immunostained slides were evaluated by light microscopy. A proportion score and intensity score was assigned, using the system described by Allred et al (17). The proportion score represented the estimated fraction of positively staining tumor cells (0 = none; 1 = <1/100, 2 = 1/100-1/10; 3 = 1/10-1/3; 4 = 1/3-2/3; 5 = >2/3). The intensity score represented the estimated average staining intensity of positive tumor cells (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The overall amount of positive staining was then expressed as the sum of the proportion and intensity scores (ranges = 0 for negative staining and 2-8 for positive staining).

Plasmids: The OPN plasmid used for generation of riboprobes (OP-10) consisted of the complete protein-encoding region of human osteopontin (1493 bp) cloned into a Bluescript SK vector at the *Eco*RI site between the T3 and T7 promoters, with the 5' end of the gene downstream to the T3 promoter (18).

Riboprobes: Riboprobes were generated by *in vitro* transcription from linearized templates with the appropriate phage RNA polymerase (Promega Corp., Madison, WI) in the presence of digoxigenin-UTP (Boehringer Mannheim, Montreal, PQ). Antisense riboprobes for OPN were generated by transcription from the T7 promoter of plasmid OP-10, and negative control sense riboprobes by transcription from the T3 promoter.

In situ hybridization: ISH was performed essentially as described previously (19). Briefly, four micron paraffin sections were cut under RNase-free conditions onto Superfrost Plus (Fisher, Ottawa, ON) slides, dewaxed in xylene, and rehydrated. Permeabilization was performed by treating at room temperature sequentially with 0.2M HCl, 0.2% Triton X-100 in PBS, and 40 μg/ml proteinase K for 10 min each. Slides were then washed in 0.1X PBS, refixed for 30 min at room temperature in 4% paraformaldehyde, washed again in 0.1X PBS and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform for 15 min, followed by treatment with absolute ethanol for 5 min and 95% ethanol for 15 min, and then air-dried. Probes were diluted in prehybridization mix to a concentration of 800 ng/ml, and 200 μl of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, washed in 0.2X SSC at 55°C for 30 min, rinsed in RNase buffer (0.5M NaCl, 10mM PIPES [pH 7.2], 0.1% Tween 20) at room temperature for 10 min, and incubated in 20 μg/ml RNase A (Sigma, St. Louis, MO) for 30 min at 37°C to remove unbound single-stranded RNA. Slides were washed in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature for 10 min and blocked with 3% normal sheep serum in buffer 1 at room temperature for 30 min. To detect specific hybrids, slides were

incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) (1 to 1,000 dilution in buffer 1 with 3% normal sheep serum) overnight at 4°C, then washed twice (10 min each) with buffer 1, and twice (5 min each) in buffer 2 (100 mM Tris-HCl [pH9.5], 100 mM NaCl, 50 mM $MgCl_2$). Hybrids bound to anti-digoxigenin antibody were then visualized by a color reaction containing nitroblue tetrazolium salt (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Levamisole (0.24 mg/ml) in buffer 2. (An alkaline phosphatase substrate kit IV [BCIP/NBT] [Vector Laboratories, Burlingame, CA] was used). Color was allowed to develop for 4-6 hours in the dark. Slides were then dehydrated, washed in xylene, mounted with permount, and viewed and photographed by a light microscope. Positive areas showed brown-purple cytoplasmic staining.

Controls for specificity of probe binding included: (a) hybridizing with sense riboprobes and (b) omitting riboprobe entirely. Other controls included antisense and sense probe testing of cell blocks prepared of a cell line known to synthesize high levels of OPN (MDA-MB-435 cells [12]).

Statistical Analyses: Univariate associations between all reported risk factors and outcomes were first assessed using chi-square tests, Fisher's exact test, or logistic regression where appropriate. All variables with a univariate p-value ≤ 0.25 were considered eligible for inclusion in the multivariate model (20). A multiple-step backward selection method was then used and variables were removed from the model if significance fell above a p-value of 0.05. Those variables remaining in the model were considered to be independent predictors of outcome. All analysis was performed using SAS for Windows, version 6.08 (21).

RESULTS

Patient ages in the study group ranged from 26 to 83 years, with a mean of 52.1 years. Fifty-three percent of the patients were premenopausal, and 47% were postmenopausal. Tumor sizes ranged from 0.6 to 8.0 cm (mean=2.5 cm). Of those tumors in which the biochemical hormone receptor status was known (93% of tumors), 70% were ER positive, and 74% were PR positive. The pathology of all tumors was reviewed by us (FPO, ABT). One hundred and thirty-six of 143 invasive tumors were of "no special type" (infiltrating ductal carcinoma, not otherwise specified). The special type carcinomas included two tubular carcinomas, a tubulolobular carcinoma, two classic type of infiltrating lobular carcinomas, a pleomorphic variant of infiltrating lobular carcinoma, and a mucinous carcinoma. Thirty patients (21%) had grade I carcinomas, 55 patients (38.5%) had grade II carcinomas, and there were 58 (40.5%) grade III tumors. In addition, there were 11 of 154 cases composed predominantly of high grade (comedo-type) ductal carcinoma *in situ* with microinvasion (defined as invasive component $<$3mm) (7.1%). Using a previously described semiquantitative method of scoring p53 immunopositivity, and cutpoint analysis to determine the optimum cutoff score (17), 22 of the 154 cases (14.3%) showed p53 positivity.

Immunohistochemical staining of sections from the 154 tumors showed diffuse cytoplasmic positivity for OPN in scattered tumor infiltrating macrophages and/or lymphocytes in the majority (70%) of cases. Accumulation of extracellular and macrophage-associated OPN was also seen in regions of necrosis and calcification, as previously described (8). Benign epithelium showed only focal, faint staining for OPN in occasional sections. When present, OPN positivity in benign epithelium was usually seen concentrated at the luminal (apical) surface

of the cells. Forty tumors (26%) showed OPN staining of the carcinoma cells themselves, with immunohistochemical scores ranging from 3 to 7. The OPN positivity was seen mainly in an intracytoplasmic (often perinuclear), or less commonly in a cell surface distribution in the neoplastic cells. It is this subgroup of tumors (showing OPN positivity of the tumor cells themselves) that showed the most promise for examination of OPN as a prognostic indicator. Cutpoint analysis was performed for tumor cell OPN staining, and the optimum cutoff score was >4. Using this cutoff score, 11 (7%) of tumors showed OPN tumor cell positivity. A representative tumor showing OPN tumor cell positivity is shown in Figure 1.

Patients were followed for a median of 7 years, range 1.7 to 16.3. Forty five patients died of disease. Univariate survival analysis showed tumor cell OPN positivity (score >4) to be significantly associated with both decreased disease free survival (DFS) and overall survival (OS) (Figure 2, Table 1). In a multivariate model, which included patient age, menopausal status, tumor size, grade, hormone receptor status, and p53 positivity (determined by immunohistochemistry), tumor cell OPN positivity remained a significant predictor of decreased overall survival ($p=0.0138$, $RR=2.971$), but not of disease-free survival ($p=0.3217$, $RR=1.634$). These data thus indicate that in this group of LNN patients, immunodetectable OPN present within the tumor cells themselves may have value as an independent prognostic indicator.

In situ hybridization was performed in order to determine the cellular distribution of OPN mRNA expression in sections from selected immunopositive tumors (all infiltrating mammary carcinomas of "no special type" (ductal), 4 showing high and 4 low level of tumor cell immunopositivity for OPN). Non-neoplastic ductal epithelium in these sections showed only

focal, faint staining. In all cases and as described previously (7,8), OPN mRNA was detected in scattered groups of tumor infiltrating macrophages and lymphocytes (Figure 3). In addition, however, OPN mRNA expressing invasive carcinoma cells were also seen, both as isolated cells and variably-sized clusters. The degree of OPN mRNA expression by the tumor cells was heterogenous and regional. Strong focal positivity for OPN mRNA was seen in the invading cells of tumors that had shown strong immunopositivity of the tumor cells. Although difficult to accurately quantify, it appeared from matched sections that more of the tumor cells stained positive for OPN protein by IHC than were positive for OPN mRNA by *in situ* hybridization. This could either be a phenomenon related to difference in sensitivity of detection, or alternatively may suggest that OPN protein present in many of the cells had been acquired either from the subpopulation of cancer cells that were actively expressing OPN, or from tumor infiltrating macrophages and lymphocytes. The MDA-MB-435 control cells, which are known to express high levels of OPN (12), showed strong positive staining when hybridized with antisense OPN riboprobe, and complete lack of staining with sense riboprobe (Figure 3). None of the tumors showed positive staining when hybridized with sense riboprobe, or when the riboprobe hybridization step was omitted entirely.

DISCUSSION

This study is the first to demonstrate that human breast carcinoma cells themselves (in addition to tumor infiltrating macrophages and lymphocytes) can synthesize OPN *in vivo*, and that immunohistochemical **tumor cell** positivity for OPN is associated with poor clinical outcome in a group of LNN breast cancer patients. We found that tumor infiltrating macrophages and

lymphocytes that are positive for both OPN mRNA (by ISH) and protein (by IHC) are present both in tumors that do and do not show evidence of tumor cell immunopositivity for OPN. Thus, the mere presence of OPN positive inflammatory cells does not determine the presence of tumor cell OPN. This distinction is important, as we have shown in this pilot study that OPN present in or on the tumor cells themselves is a significant independent prognostic indicator in lymph node negative disease.

Results from cell culture have shown expression of OPN by a number of different transformed mammary epithelial cell lines (eg. D2HAN cells [10,11], MDA-MB-435 cells [12], 21T series [22] cell lines [Tuck et al., unpublished observations]). It is thus reasonable to expect that at least in some instances, breast cancer cells themselves may synthesize OPN *in vivo* as well. In the present study, we have shown that indeed, regional expression of OPN mRNA by the tumor cells themselves may be found in immunopositive tumors, in addition to expression by tumor infiltrating macrophages and lymphocytes. Although the ISH procedure does not allow for accurate quantitation of expression, it would appear from assessment of matched sections that there were fewer OPN mRNA positive than OPN immunopositive tumor cells. This could either be due to a difference in sensitivities of the procedures (ISH vs. IHC), or to the fact that some of the tumor cells were not actively synthesizing OPN, but instead acquired the protein by uptake from another source (either tumor-infiltrating inflammatory cells or a subpopulation of tumor cells over-expressing OPN). This finding stands in contrast to reports of OPN mRNA expression exclusively in tumor infiltrating macrophages and lymphocytes (7,8). We believe this difference is most likely due to a sampling phenomenon. In both our work, and one of these previous studies (7), the majority of tumors show the presence of tumor infiltrating macrophages

and/or lymphocytes which stain for OPN protein by IHC, whereas only a minority show **tumor cell** positivity for OPN protein by IHC. The tumor samples which we selected for screening for ISH were derived from a bank of 154 tumor samples. From these, four of the tumors chosen for ISH testing were those showing the strongest immunopositivity of the tumor cells themselves. Other studies (7,8) have examined a smaller number of breast cancers, and did not report specifically selecting those high in tumor-cell OPN immunopositivity for ISH analysis.

Our demonstration of an association between tumor cell OPN and outcome suggests that at least in this group of LNN patients, those cancers containing tumor cells positive for OPN behave differently than those that do not. OPN-positive tumor cells may be different through some combination of the ability to sequester the molecule from the environment (perhaps by expression of specific cell surface receptor(s) necessary for OPN accumulation), and the ability to themselves sythesize the molecule. Although the functional consequences of OPN on breast cancer cells have not yet been completely elucidated, there is evidence from cell culture that at least some breast cancer cells can adhere to and show increased migration in response to OPN (12-15), suggesting at least one potential mechanism for the increased aggressive behavior of OPN-positive tumors.

In summary, this pilot study shows an association between tumor cell immunopositivity for OPN and outcome in a group of 154 LNN breast cancer patients, and supplies evidence that at least some of the tumor cell OPN may originate from the tumor cells themselves. This work suggests potential value of OPN as an independent prognostic indicator in LNN breast cancer, and indicates the need not only for definitive clinical study, but also for a more detailed analysis of the biological effects of OPN on breast cancer cells, with a view towards the future

identification of new therapeutic targets.

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Table 1: Tumor Cell OPN (score >4) in Univariate (Cox Proportional Hazards) and Multivariate (Backwards Elimination Method) Analysis as a Prognostic Indicator for Patients with Invasive, Lymph Node Negative Breast Cancer

	DFS	OS
Univariate	p=0.0025	p=0.0294
Multivariate	p=0.3217	p=0.0138
Risk Ratio	1.634	2.971

FIGURE LEGENDS

Figure 1: Immunohistochemical positivity of tumor cells for OPN protein (score 6) in an infiltrating mammary carcinoma of no special type, combined histologic grade II/III (original magnification, X400).

Figure 2: Disease free survival (DFS) (a) and overall survival (OS) (b) curves for lymph node negative breast cancer patients whose tumors were OPN-positive [B] (**tumor cell** IHC score >4) vs. OPN-negative [A] (**tumor cell** IHC score ≤ 4), as determined by immunohistochemistry (DFS $p=0.0025$; OS $p=0.0294$).

Figure 3: *In situ* hybridization for OPN mRNA. a: Strong cytoplasmic positivity for OPN is seen regionally in carcinoma cells of OPN immunopositive tumors. b: Tumor associated mononuclear inflammatory cells showing strong cytoplasmic staining for OPN mRNA. c: Negative control, showing lack of cytoplasmic staining for OPN mRNA in cultured human MDA-MB-435 cells (known by Northern analysis to express high levels of OPN mRNA) when probed with "sense" riboprobe (phase contrast). d: Positive control, showing strong cytoplasmic staining for OPN mRNA when cultured human MDA-MB-435 cells are probed with "antisense" riboprobe. (Original magnification panels a-d, X400).

Figure 1

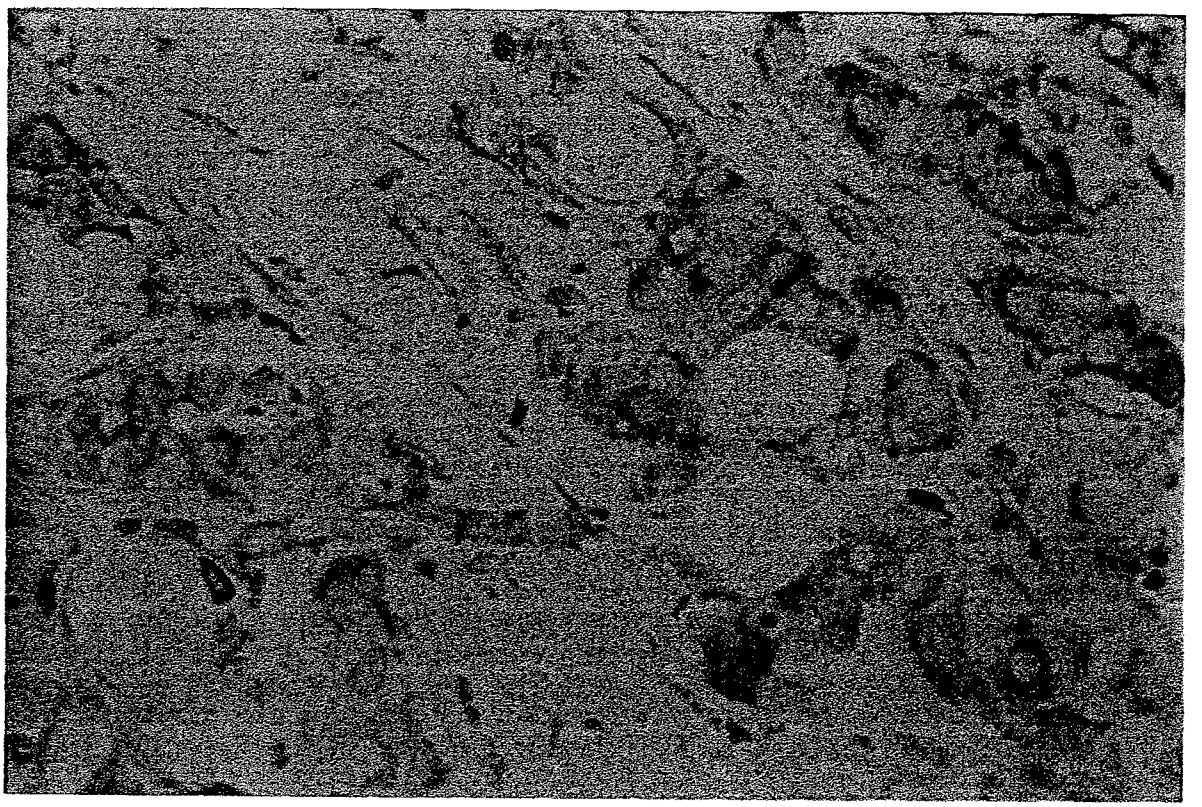


Figure 2

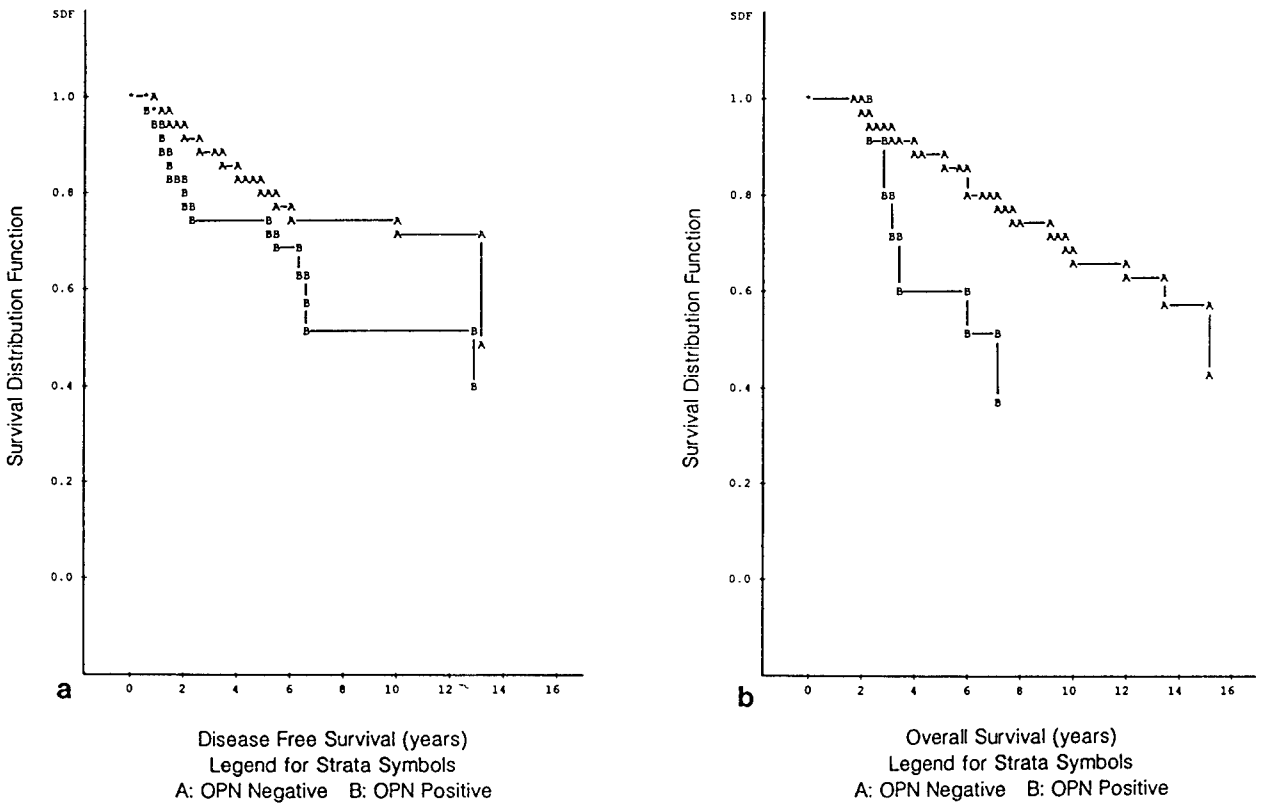
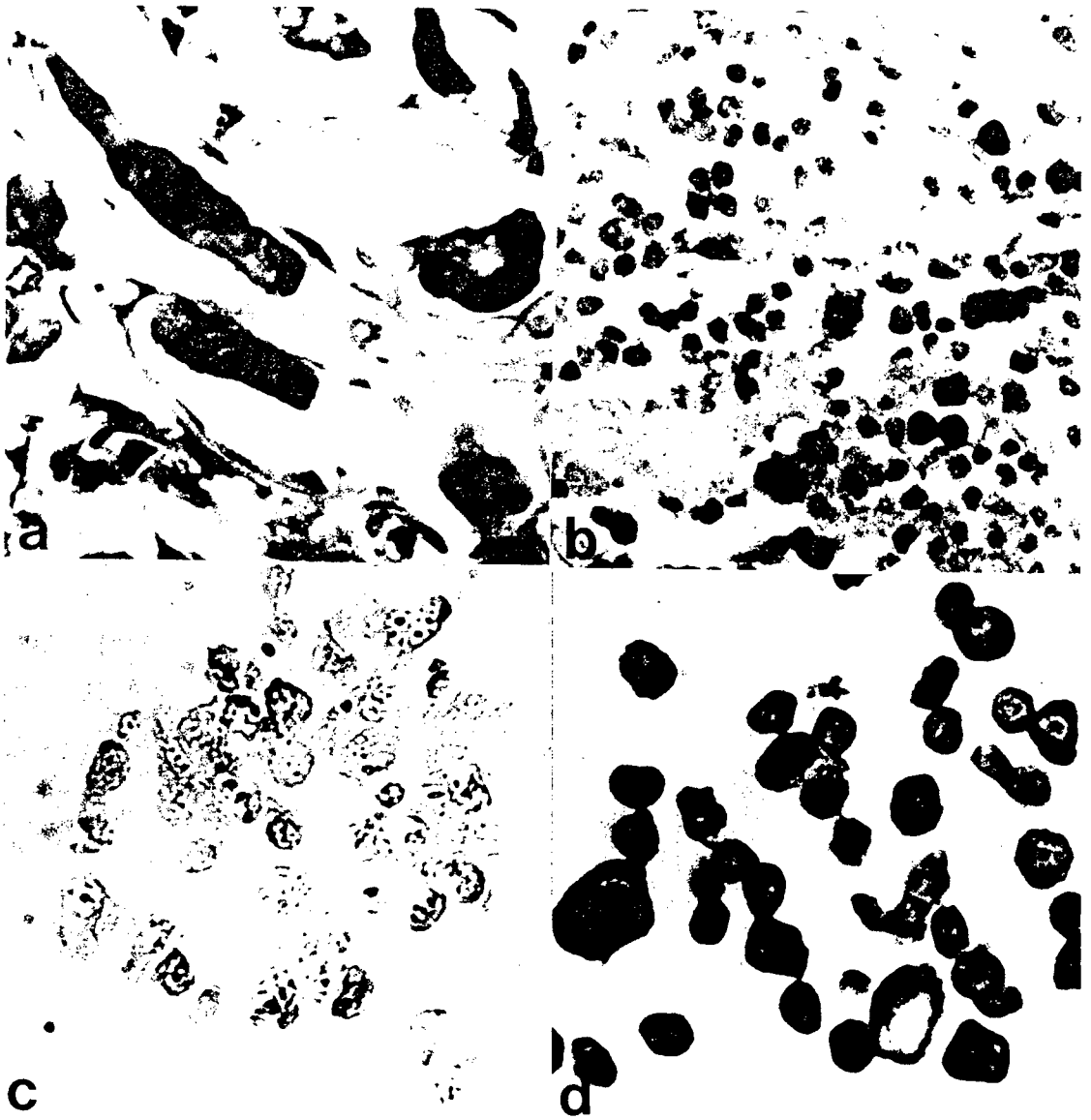

SDF
Survival Distribution Function
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
a
Disease Free Survival (years)
Legend for Strata Symbols
A: OPN Negative B: OPN Positive
SDF
Survival Distribution Function
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
16
b
Overall Survival (years)
Legend for Strata Symbols
A: OPN Negative B: OPN Positive

Figure 3



OSTEOPONTIN (OPN) AND P53 EXPRESSION ARE ASSOCIATED WITH TUMOR PROGRESSION IN A CASE OF SYNCHRONOUS, BILATERAL, INVASIVE MAMMARY CARCINOMAS

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ABSTRACT

Objective.-To examine the association between expression of OPN, p53, other molecular markers (Ki67, c-erbB2, and estrogen receptor protein [ERP]) and tumor progression in a case of synchronous, bilateral invasive mammary carcinomas of the same histology.

Design.-Immunohistochemistry was performed on formalin fixed, paraffin embedded tissue sections. Plasma OPN level was determined by a quantitative antigen capture assay.

Setting.-The patient was seen, treated and followed over a five year period at the London Regional Cancer Centre.

Patient.-At age 60, presented with bilateral infiltrating mammary carcinomas of the same histologic type and grade. Bilateral mastectomy and axillary node dissection showed involvement of 3/12 right axillary and 0/11 left axillary nodes. She later developed a right chest wall recurrence, followed by widespread metastatic disease (to skull, liver, and left femur).

Results.-The right-sided breast primary was OPN and p53 positive, while the left-sided tumor was negative for both. The development of right axillary lymph node metastases, chest wall recurrence, and distant metastases was associated in all instances with the IHC profile of high level expression of OPN and p53. Plasma assay for OPN at the time of last admission showed a markedly elevated OPN level.

Conclusions.-In keeping with previous reports (1-9), increased p53 expression was found to be associated with increased aggressiveness. The association of increased OPN expression with increased malignancy in breast cancer is a novel finding, and raises the possibility of a role for OPN in tumor progression, and the potential for this marker in predicting clinical aggressiveness.

INTRODUCTION

In recent years, there has been an intensive search for molecular markers of tumor aggressiveness in breast cancer. In addition to hormone receptor status, some candidate molecules showing promise in this regard include c-erbB2 (9-18), Ki67 (19-22), and p53 (1-9).

p53 mutations appear to represent the most common cancer-related change at the gene level (23). Over-expression of p53 (most often associated with point mutations) has been found in 16% to 52% of sporadic breast cancers and is associated with a poor prognosis (1-3). Immunopositivity for p53 has been found to be an independent prognostic indicator in both lymph node negative (4-6,8,9) and lymph node positive (2,4,7,8) patients.

Osteopontin (OPN) is a secreted phosphoprotein which is expressed by a limited number of normal cells and tissues (including lactating mammary gland, developing bone, kidney, activated T-cells and macrophages, and smooth muscle cells) (reviews 24,25). OPN has also been identified by several groups as a tumor-associated protein (review 26), and its expression has been linked to the malignancy of ras-transformed fibroblasts (27-29). Recently, OPN has been shown to be expressed by human mammary carcinoma (MDA-MB-435) cells (30,31). OPN has been shown to mediate an RGD-inhibitable cell-substrate attachment in these cells, as well as chemotaxis in a cell migration assay (30,31). In the clinical setting, there have been reports of increased plasma levels of OPN in human breast cancer (32-34), and of increased OPN in primary breast tumors (35-37). The significance of increased OPN expression (either plasma or tumor levels) in predicting the biological behavior of a tumor is unknown at present, but is of great potential clinical impact in prognostication and planning of treatment strategies.

We report here a case of synchronous, bilateral infiltrating mammary carcinomas showing a very similar histologic appearance, but each with a distinct immunohistochemical profile. Lymph node and distant metastases were uniformly found to be associated with positivity for both p53 and OPN. Plasma levels of OPN were significantly elevated over an established normal range, suggesting that level of OPN in either plasma or primary tumor may be predictive of propensity for metastasis.

REPORT OF A CASE

A case of bilateral, synchronous invasive mammary carcinoma of No Special Type [NST] (invasive ductal carcinoma, NOS) is reported, in which immunophenotyping for OPN and p53, as well as a number of other markers was performed. The natural course of the two primary tumors seemed quite different, one behaving in a more indolent, and the other in a more aggressive fashion. Increased levels of OPN and p53 expression (as determined by immunohistochemistry [IHC]) were associated with the more aggressive tumor and its metastases.

The woman here described presented first at 34 years of age with a 0.5 cm, hard nodular mass of the left breast. This mass was biopsied and found to represent a non-specific giant cell granulomatous inflammation, with no evidence of malignancy, no evidence of proliferative breast disease (with or without atypia) and no identifiable infectious cause. She next presented 26 years later (age 60) with a mammographic lesion in each breast. Needle localization biopsy of both lesions showed both to be gritty and stellate on gross examination, measuring 2.5 cm in greatest dimension on the right, and 2.3 cm on the left. Sections of both masses showed infiltrating mammary carcinoma of NST, combined histologic (SBR) grade II/III. Both showed an associated solid and cribriform, intermediate and focal high grade ductal carcinoma *in situ* (DCIS), with involvement of ducts both within and away from the region of involvement by invasive carcinoma (Extensive Intraductal Component [EIC] positive). Furthermore, both showed microscopic evidence of lymphatic channel invasion (without evidence of vascular invasion), and both were estrogen receptor (ER) positive.

The following month, the patient returned for bilateral simple mastectomy and axillary node dissection. Examination of the right breast tissue showed residual DCIS, without evidence of residual invasive carcinoma. The left breast tissue showed no evidence of residual malignancy. Examination of the axillary lymph nodes showed involvement of 3/12 right axillary and 0/11 left axillary nodes. Adjuvant treatment was initiated at this time, involving six cycles of CMF.

Four years following the initial diagnosis of malignancy, the patient presented with a right chest wall recurrence. The following year she was found to have developed widespread metastatic disease,

including skull and liver metastases, as well as a subcapital pathologic fracture of the left femoral head, for which she underwent a total hip arthroplasty. The histology of both the chest wall and femoral head metastasis was that of a poorly differentiated metastatic adenocarcinoma, consistent with a breast primary. The femoral head metastasis was found to be ER negative by immunohistochemistry. Further treatment included radiotherapy to the left femoral head and skull, as well as a single cycle of CEF chemotherapy. The patient died of her disease several months later the same year.

MATERIALS AND METHODS

IHC was performed on 4 micron sections of routinely fixed (10% formaldehyde), paraffin embedded tissues. The sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 minutes. An antigen retrieval method was used on all slides prior to immunostaining. This involved heating tissue sections in citrate buffer (10mM, pH 6.0) in a microwave oven (600 watt) for 7 minutes. Immunostaining was performed using a streptavidin-biotin complex method (Zymed histostain kit-Dimension Laboratories, Toronto, Ont.). Non-specific staining was blocked by incubating slides with 5% normal goat serum. The following antibodies were used: anti-human osteopontin (OPN) (monoclonal antibody mAb53 against the recombinant GST-human OPN fusion protein [31]), anti-human p53 (monoclonal antibody DO7, DAKO Corp., CA, 1:50), anti-human Estrogen Receptor Protein (ERP) (monoclonal antibody ER1D5, DAKO Corp., CA, 1:50), anti-human c-erbB2 (monoclonal antibody CB11, Novacastra Laboratories, U.K., 1:40), and anti-human Ki67 (polyclonal antibody A047, DAKO Corp., CA, 1:50). For each antibody, the slides were incubated for one hour at room temperature. Slides were then rinsed with PBS, and a biotinylated secondary antibody was applied for 15 minutes, followed by a PBS rinse and treatment with the streptavidin-enzyme conjugate for 10 minutes. The chromogen used was aminoethyl carbazol (reddish-brown signal), and slides were counterstained with Mayer's hematoxylin. Immunostained slides were evaluated by light microscopy by two independent observers. A proportion score and intensity score was assigned for each antibody, using the semiquantitative system described by Allred et al. (5). The proportion score represented the estimated fraction of positively staining tumor cells (0 = none; 1 = <1/100, 2 = 1/100-1/10; 3 = 1/10-1/3; 4 = 1/3-2/3; 5 = >2/3). The intensity score represented the estimated average staining intensity of positive tumor cells (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The overall amount of positive staining was then expressed as the sum of the proportion and intensity scores (ranges = 0 for negative staining and 2-8 for positive staining) (reported as total score Observer 1; Observer 2).

As a negative control in each case, the IHC procedure was performed with the omission of the primary antibody. Positive controls in each case included tissue sections previously known to express high

levels of the test antigen. Pattern of staining also provided an indirect internal control for specificity of staining (i.e. nuclear for Ki67, p53 and ERP, cytoplasmic for OPN, membranous for c-erbB2). An added control for OPN involved IHC staining of cell pellets of MDA-MB-435 cells (a human breast carcinoma cell line known by Western analysis and ELISA assay to express high levels of OPN [30]).

Plasma level of OPN was determined at the time of the last admission by a quantitative antigen capture sandwich assay (38). In this approach, the monoclonal antibody (mAb53) against OPN is adsorbed to plastic and acts as an antigen capture reagent during the first incubation with solutions of human plasma or known standard dilutions of human OPN. In the second incubation, a rabbit polyclonal antibody against human OPN is added. In the third step, biotinylated goat anti-rabbit antibody is added, followed by streptavidin-alkaline phosphatase to prepare for the signal development with substrate. Plasma level was compared with reference standards from normal women (negative controls) (38).

RESULTS

In spite of the histologic similarity of the right and left breast primary tumors (see Figure 1), their immunohistochemical profile differed (Table 1). Both were negative for c-erbB2 expression, in contrast to strong positivity found in a comedo-type carcinoma *in situ* control (not shown). Each of the primary tumors was ER positive, and each showed comparable positivity for the cell proliferation marker, Ki67. In contrast, the two tumors were quite different for p53 and OPN expression. Nuclear staining for p53 was quite strong (6;6) in the malignant cells of the right breast primary (both *in situ* and invasive), whereas it was undetectable (0;0) in those of the left breast primary. Cytoplasmic positivity for OPN was seen in the malignant (*in situ* and invasive) cells of the right breast primary (5;4), but was undetectable in those from the left (0;0). Scattered infiltrating macrophages and lymphocytes showed positivity for OPN in both primary tumors. Tumor cell positivity for p53 (Figure 2) and OPN (Figure 3) was also found in the later right chest wall recurrence, and at the metastatic sites, including right axillary lymph nodes and bone. The highest level of expression of both was found at the most remote site (the left femoral head) (p53: 8;7, OPN: 6;6), at the latest point in time following the initial diagnosis (suggesting the possibility of tumor progression). A parallel decrease in ERP expression was seen at chronologically more advanced stages, with clinically insignificant levels of expression (i.e. essentially ER negative) in the malignant cells of the femoral head metastasis. Ki67 expression was examined both by the semi-quantitative scoring system described above (Table 1), and by determination of labelling indices (not shown). Both systems showed the same rank order of labelling, with equal positivity of the right and left primary tumors, a similar level of staining in the lymph node metastases, and a greater amount of labelling in both the chest wall recurrence, and the bone metastasis.

At the time of the last admission (for widespread metastases, with femoral head involvement and subcapital pathologic fracture), the plasma level of OPN was 324 ng/ml (normal reference range 14-64 ng/ml). This value is well above the 95th percentile as determined for normal women (50 ng/ml) (38), and is within the predicted range for patients with metastatic disease (34). From our previous prospective clinical study, given this level of plasma OPN, we would have predicted a median survival of 8 months

(34). Survival in this case was indeed only a few months following this admission.

COMMENT

Instances of bilateral, synchronous breast cancer offer the unique opportunity to study aspects of the cellular and molecular biology of tumours under the same (or very similar) environmental conditions, at the same point in time. When both are then treated in the same fashion, at the same point in time, at the same institution, conditions may be standardized for followup. In addition, where prolonged followup is obtained, a clinicopathologic description of tumor progression with time is made possible.

In this case, we were able to follow the immunohistochemical profile of synchronous, bilateral invasive mammary carcinomas, as well as of the subsequent recurrence and metastases. Interestingly, although the histologic appearance of the two primary tumors was very similar, and although many of the molecular markers examined showed similar levels of expression, p53 and OPN expression were found to be quite different. Both were detected in the cells of the right-sided primary tumor and not detected in cells of the left-sided primary. Similarly, both were consistently found at elevated levels in the later lymph node metastases, in the right chest wall recurrence, and in cells of the bony metastasis. Further, the highest levels of expression for both were seen at the most remote site (the bony metastasis), at the latest point in time. This would suggest that the regional recurrence and metastases not only most likely arose from the right breast primary (expressing elevated levels of p53 and OPN), but that with tumor progression, selection resulting in increased expression for both may have occurred. In parallel to this, a decreased level of expression of ERP was found in cells of the lymph node metastases and chest wall recurrence, with lowest levels in cells of the bone metastasis. Similarly, higher levels of Ki67 expression were seen in the right chest wall and bone metastasis than in the primary tumors or regional lymph node metastases. However, as both Ki67 and ERP were expressed at similar levels in both right and left breast primaries, neither of these markers would have been useful in predicting the relative aggressiveness of the tumors at that point in time (in contrast to both p53 and OPN which were differentially expressed in the primary tumors).

In the case of p53, the finding of an association between level of p53 protein expression and degree of aggressiveness of breast cancer is not novel (1-9). p53 abnormalities (mutation, and hence

prolonged protein half-life and increased protein levels) have been associated with features such as large size, high proliferative fraction, high nuclear grade, and estrogen receptor negativity in primary breast cancer (5,8), and a poor prognosis (2,4-9). In addition, p53 has been reported to be an independent prognostic marker in both axillary node-negative and node-positive cases, and it has been suggested that this may make it useful in the selection of patients for adjuvant therapy (2,4-9). In the present case, the patient would have received chemotherapy regardless, on the basis of lymph node positivity, but it is interesting to speculate whether such patients (showing high levels of p53 expression) would benefit from a more aggressive chemotherapy regimen, as has been suggested in the case of high c-erbB2 expression (39).

Although a few studies have reported detectable OPN in breast carcinoma cells (30,31,35-37), an association between level of OPN expression and degree of malignancy has not yet been shown in breast cancer (an association between elevated OPN levels and poor prognosis has recently been reported in lung carcinomas [40]). We have recent evidence (33,34) that plasma OPN levels are significantly elevated in breast cancer patients harboring metastatic disease. Whether plasma OPN levels reflect the level of expression in the primary tumor, whether determination of OPN level may predict clinical course, and how OPN might effect the biological behavior of breast carcinoma cells are at present largely unknown. With regards to the latter, there is evidence that one aspect of the biological effect of OPN on human breast carcinoma cells involves cell attachment and migration-stimulating functions mediated by GRGDS amino acid sequences in the protein (30,31). It has further been suggested that binding of OPN to cell surface (integrin) receptors may trigger transduction of cell growth or invasiveness-related signals (30).

In the present case, elevated OPN protein expression was found in the more aggressive of the two histologically similar tumors. The increased OPN was seen primarily in the cytoplasm of the carcinoma cells themselves. Although scattered macrophages and lymphocytes were also seen to stain for OPN, there was no apparent qualitative or gross quantitative difference in expression by inflammatory cells between the primary tumors themselves or between primary and metastatic sites. The highest level of expression of OPN was found in malignant cells of the most distant metastatic site, at the latest point in time, suggesting the possibility of increased expression with tumor progression. Plasma OPN determination at the time of

distant metastases was markedly elevated above the normal range. Thus, as for p53 protein expression, it appears that in this case, tumor OPN expression may have predicted a more aggressive clinical course. An additional utility of OPN determination, however, may lie in the ability to readily detect increased levels in the plasma. Being a *secreted* phosphoprotein, OPN by its nature would be expected to be more readily detected in blood than a nuclear regulatory protein such as p53. The present case suggests the need for clinical studies to assess the potential role of osteopontin determination (tumor or plasma levels) as an independent prognostic indicator in human breast cancer. Work is also needed to assess changes in plasma levels of OPN with disease progression, in response to standard therapy, and perhaps following neo-adjuvant therapy, to determine if a role for OPN determination may exist in therapeutic decision-making as well.

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Table 1: Immunohistochemistry (IHC) score for selected markers* as determined by two independent observers (Observer 1; Observer 2)

Tissue	Date	OPN	p53	Ki67	c-erbB2	ERP
Left breast biopsy - benign+	Jun. 1964	0;0	0;0	2;3	0;0	5;5
Right breast primary tumor	Oct. 1990	5;4	6;6	4;4	0;0	6;6
Left breast primary tumor	Oct. 1990	0;0	0;0	5;5	0;0	7;6
Right axillary lymph node metastasis #1	Nov. 1990	3;3	6;5	4;5	0;0	4;4
Right axillary lymph node metastasis #2	Nov. 1990	5;4	6;5	4;5	0;0	4;4
Right chest wall recurrence	Mar. 1994	5;5	6;5	8;7	0;0	4;4
Bone metastasis	Jun. 1995	6;6	8;7	6;6	0;0	2;2

*: IHC score determined as described in Materials and Methods; 0 = negative, positive range 2-8.

+: IHC scores determined for benign epithelium.

FIGURES

Figure 1: Histology of the right- (a) and left- (b) sided primary breast tumors. Both are infiltrating mammary carcinomas of no special type, SBR Grade II/III. (Hematoxylin and eosin, original magnification X250)

Figure 2: Immunohistochemistry for p53. (a): Right-sided primary breast tumor showing focal strong nuclear positivity for p53 (IHC score 6;6). (b): Left-sided primary breast tumor showing negative staining for p53 (IHC score 0;0). (c): Metastatic carcinoma cells in right axillary lymph node showing regional strong nuclear positivity for p53 (IHC score 6;5). (d): Left femoral head metastasis, showing regional strong nuclear positivity for p53 (IHC score 8;7). (Original Magnification X400)

Figure 3: Immunohistochemistry for osteopontin (OPN). (a): Right-sided primary breast tumor showing focal cytoplasmic positivity for OPN (arrows, IHC score 5;4). Cytoplasmic positivity is coarsely granular, and often perinuclear in location. (b): Left-sided primary breast tumor showing negative staining for OPN (IHC score 0;0). (c): Metastatic carcinoma cells in right axillary lymph node showing focal positivity for OPN (arrows, IHC score 5;4). (d): Left femoral head metastasis, showing regional positivity for OPN (IHC score 6;6). (e): Macrophage giant cells of original (benign) left breast biopsy showing cytoplasmic positivity for OPN. (f): Benign ductal epithelium of original left breast biopsy, showing negative staining for OPN. (Original Magnification X400).

Figure 1

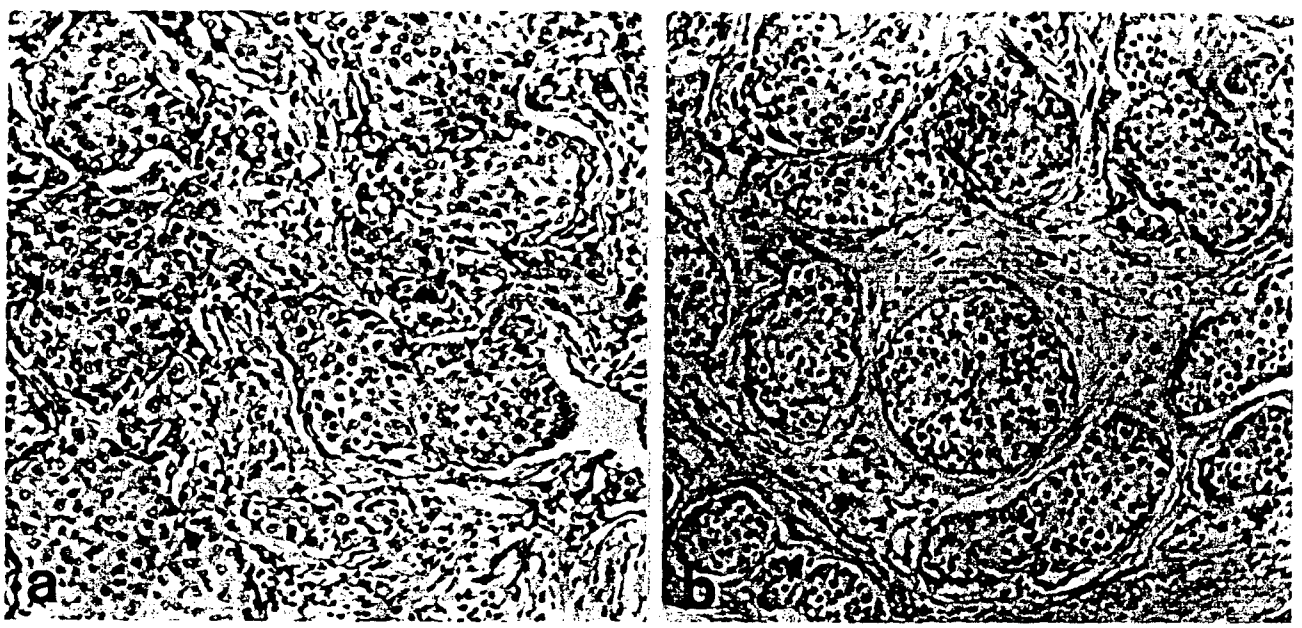


Figure 2

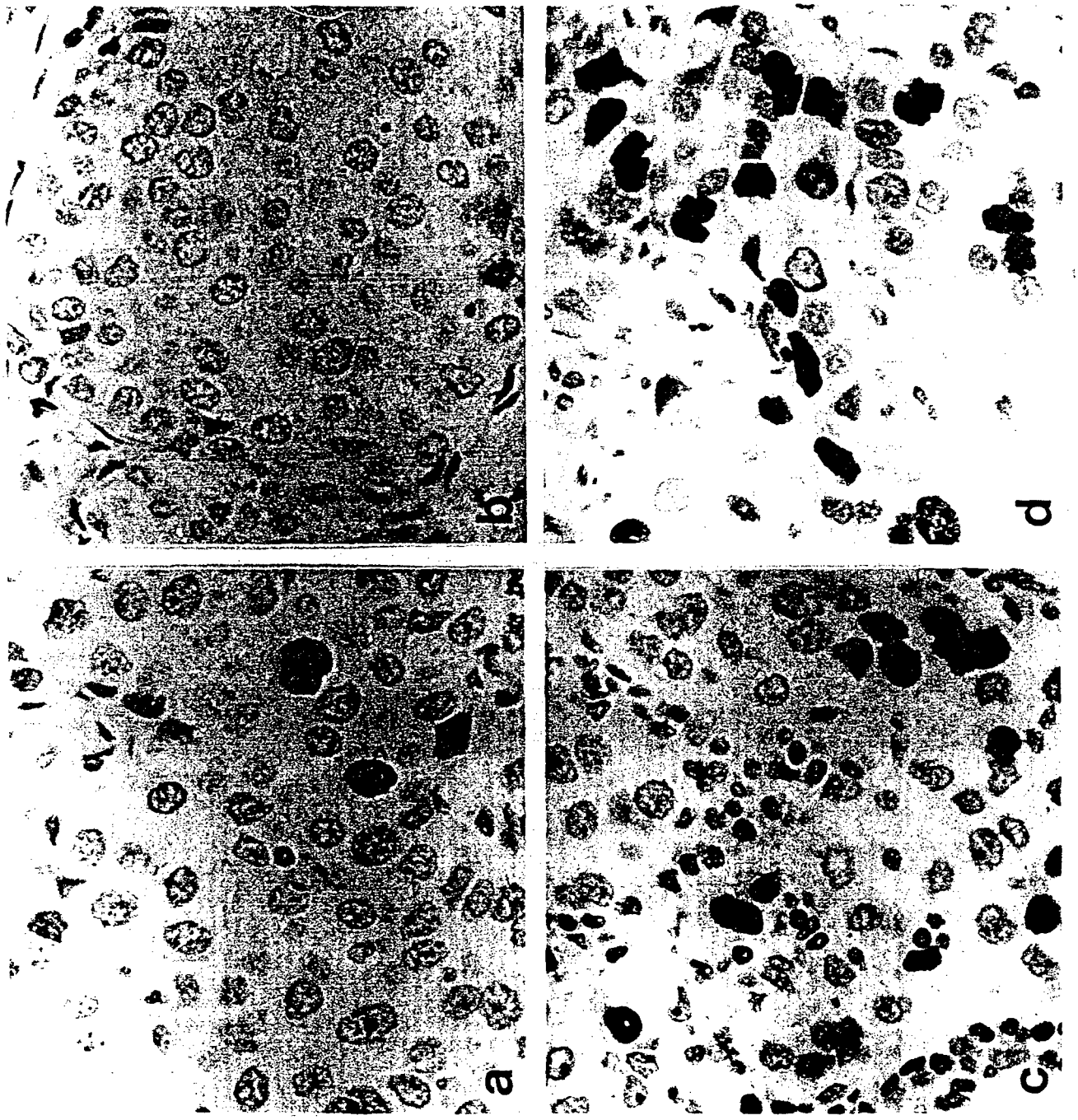
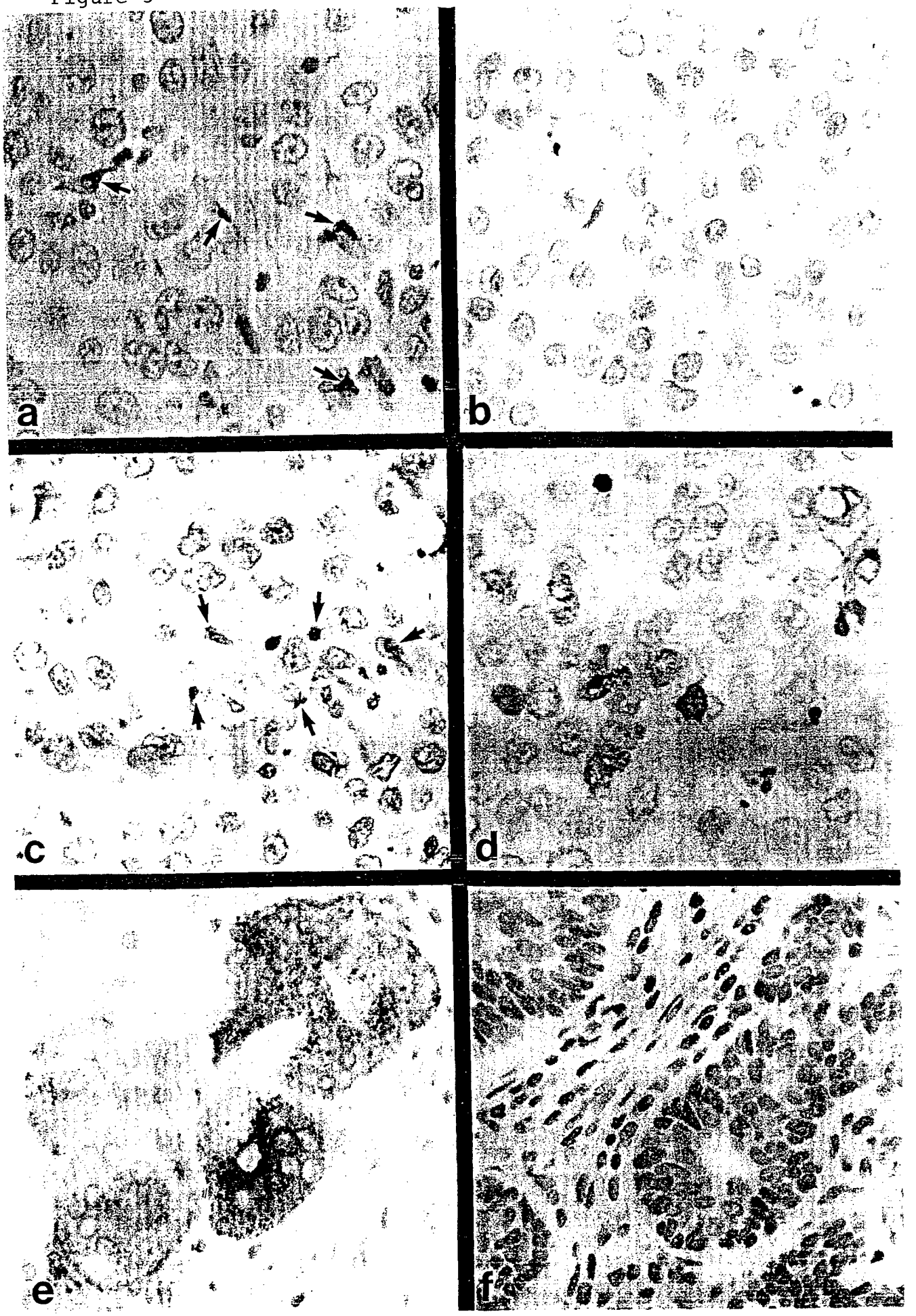


Figure 3



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Elevated Plasma Osteopontin in Metastatic Breast Cancer Associated with Increased Tumor Burden and Decreased Survival[1]

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ABSTRACT

Osteopontin (OPN) is a secreted, integrin-binding phosphoprotein that has been implicated in both normal and pathological processes; qualitative increases in OPN blood levels have been reported in a small number of patients with metastatic tumors of various kinds. We measured plasma OPN levels in 70 women with known metastatic breast carcinoma, 44 patient controls who were on follow-up after completion of adjuvant treatment for early breast cancer, and 35 normal volunteers.

The median plasma OPN of patients with metastatic disease was 142 μg/liter (range, 38–1312 μg/liter) and was significantly different ($P < 0.0001$, Mann Whitney U test) from both control groups (medians, 60 and 47 μg/liter; ranges, 15–117 and 22–122 μg/liter). Furthermore, we found that increasing plasma OPN is associated with shorter survival ($P < 0.001$) when patients were grouped in terciles for plasma OPN. This was also demonstrated when using a Cox proportional hazards model. Median plasma OPN levels were significantly increased for three or more sites of involvement (median, 232 μg/liter; $n = 13$) *versus* 1 or 2 metastatic sites (medians, 129 and 130 μg/liter; $n = 29$ and 28, respectively). Plasma OPN levels were correlated with other biochemical markers related to the extent of disease, such as serum alkaline phosphatase, aspartate succinate aminotransaminase, and albumin ($r = 0.81$, 0.62, and −0.56, respectively; all $P < 0.001$).

This study demonstrates a statistically significant elevation in plasma OPN in the majority (~70%) of a large series of patients with metastatic breast cancer when compared (95th percentile) to healthy women or patients who had completed adjuvant treatment for early-stage breast cancer. Furthermore, this is the first study to demonstrate that higher OPN levels in patients with metastatic breast cancer may be associated with an increased number of involved sites and decreased survival.

INTRODUCTION

OPN[3] is a secreted, integrin-binding phosphoprotein that has been implicated in various normal and pathological processes (reviewed in Refs. 1–4). OPN is expressed by many normal cells including various epithelial cells as well as activated macrophages. OPN is a tumor-associated protein secreted by many tumor cells in culture (reviewed in Refs. 4 and 5). In addition, OPN has been identified in a variety of types of human carcinomas, where its expression was localized primarily to macrophages (6). OPN expression was found to be higher in breast carcinomas than in benign breast lesions (7, 8). OPN also has been detected in a variety of human body fluids including blood, urine, and milk (5, 9–13).

Using Western blot analysis with polyclonal antibodies, Senger *et al.* (5) reported elevated levels of OPN in the plasma and serum of a small number of patients (10 of 13) with a variety of disseminated carcinomas. Included in that study were results from a single patient with metastatic breast cancer; no clinical data were available for the patients in that study. Further work on the significance of plasma OPN levels in metastatic cancer were hampered in part by the lack of specific antibodies to allow the development of a fast and reliable immunoassay. We generated high-avidity monoclonal antibodies to native osteopontin (14) and developed an ELISA for the quantitative measurement of OPN levels in plasma (15) and urine (12). In normal women, we found that plasma OPN levels were independent of hormonal influences of the menstrual cycle (15). Here we used this assay to quantify OPN plasma levels in 70 patients with metastatic breast cancer and appropriate control groups to determine if OPN levels are elevated in metastatic breast cancer, and if OPN levels are associated with clinicopathological findings or survival.

MATERIALS AND METHODS

Patients. This study was conducted on female patients aged >18 years who were being followed at the London Re-

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[3] The abbreviations used are: OPN, osteopontin; AST, aspartate succinate aminotransaminase.

estimates, Cox proportional hazards model, and the Martingale residuals were computed with the SAS statistics package (SAS Institute, Inc., Cary, NC).

RESULTS

We measured plasma OPN in 70 patients with metastatic breast cancer and compared these to 44 patient controls who had previously completed treatment for early-stage breast cancer and 35 healthy women. We have shown previously that OPN plasma levels in normal women have a median of 31 μg/liter (range, 14–64 μg/liter) when the assay was performed at 37°C. In the current study, the initial antigen capture step was performed at 4°C, resulting in increased sensitivity, with the advantage of requiring smaller volumes of plasma, and a systematic increase of ~1.5-fold in calculated OPN levels using the recombinant standard. Under these assay conditions, the median plasma OPN level was 47 μg/liter (range, 22–122 μg/liter) in the healthy women's group and 60 μg/liter (range, 15–117 μg/liter) in the patient control group. The median of patients with metastatic disease was 142 μg/liter (range, 38–1312 μg/liter) and was significantly different ($P < 0.0001$, Mann Whitney U test) from that of both control groups.

The histogram of the OPN values in the study population and controls is shown in Fig. 1. The plasma OPN levels in women with metastatic breast cancer were not normally distributed and showed a very skewed distribution to large values (~30-fold range); only the distribution of the patient control group appeared to be Gaussian. The distributions for the control groups showed no significant statistical differences between the healthy volunteers and the patient control group. Sixty-nine % (48 of 70) and 71% (50 of 70) of patients with metastatic breast cancer had plasma OPN values greater than the 95th percentile of the distribution of healthy women (101 μg/liter) and patient controls (91 μg/liter), respectively.

We tested whether the survival of patients with metastatic breast cancer was related to the OPN plasma level. Fig. 2 shows that the Kaplan-Meier adjusted survival estimates of patients grouped into three according to tercile OPN levels (lower, middle, and upper thirds with OPN ≤117, 118–203, and >203 μg/liter, respectively) differed significantly ($P < 0.001$). In this cohort of 69 patients, we had a minimum follow-up period of 14 months. According to these Kaplan-Meier adjusted survival curves, the median survival was ~650 (extrapolated), 420, and 170 days for OPN values from the lower, middle, and upper thirds, respectively. Thus, the survival curves based on tercile OPN categories suggest that increasing OPN levels are associated with decreasing survival. Using a Cox proportional hazards model treating OPN as a continuous variable, there was strong evidence of an association between increasing OPN and decreasing survival ($\chi^2 = 20$, $P < 0.0001$). We found no evidence of a threshold effect of OPN when we evaluated the graphical pattern of Martingale residuals obtained from the Cox proportional hazards model (data not shown). In addition, the median plasma OPN level differed significantly ($P < 0.002$) between the patients living (128 μg/liter) and those who died (203 μg/liter) during follow-up. Thus, increased plasma OPN levels were significantly associated with shorter survival using a variety of statistical analyses.

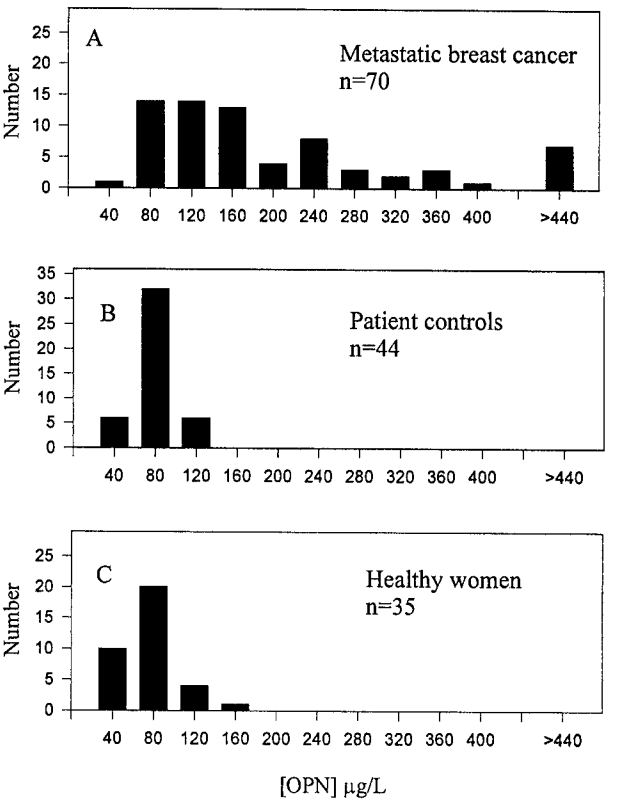

Fig. 1 Frequency distribution of plasma OPN in women diagnosed with metastatic breast cancer, treated for primary breast cancer, and normal controls. The average OPN level was calculated from plasma samples using the immunoassay described in "Materials and Methods" and displayed as a histogram with the upper bound of each interval on the *abscissa*. *A*, frequency distribution of women diagnosed with metastatic breast cancer: $n = 70$, median OPN level is 142 μg/liter, range is 38 to 1300 μg/liter. *B*, women treated for primary breast cancer: $n = 44$, median OPN is 60 μg/liter, range is 15 to 117 μg/liter. *C*, normal women: $n = 35$, median OPN is 47 μg/liter, range is 22 to 122 μg/liter. The normality test (Kolmogorov-Smirnov) indicated that only the distribution for the breast cancer-treated group was normal. The Mann-Whitney U test indicated that the median of the breast cancer metastasis group differed from the two control groups ($P < 0.001$); however, the medians of normal women and breast cancer-treated groups did not differ ($P > 0.1$).

We examined whether plasma OPN levels were related to the total number of organ sites involved or to the site of metastasis. The median OPN level was 129 μg/liter (range, 63–556; $n = 29$), 130 μg/liter (range, 50–1109; $n = 28$), and 232 μg/liter (range, 92–545; $n = 13$) with one, two, or three or more organs with metastases, respectively. The difference in median OPN levels in patients with three involved sites compared with either two or one metastatic site was statistically significant ($P < 0.05$, ANOVA on ranks). This result suggests that OPN plasma levels may be an indicator of the extent of disease because patients with multiple metastatic sites would be expected to have a higher tumor burden than patients with only a single affected site. We also found that the plasma OPN levels in 29 patients with a single organ site involvement did not differ significantly between subgroups divided according to site of involvement. Median OPN values of patients with bone ($n =$

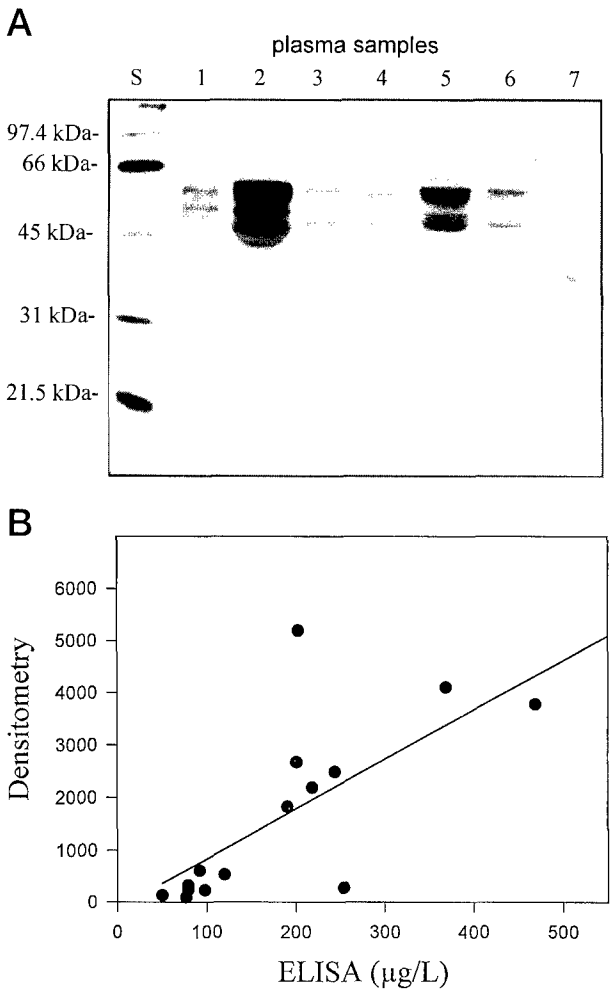

Fig. 4 Western blot analysis of plasma OPN and comparison between ELISA and densitometry for quantification of plasma OPN levels. Plasma OPN was immunoadsorbed to mAb53-conjugated Sepharose, eluted, and fractionated in 12% SDS-PAGE as described in "Materials and Methods." *A*, representative plasma samples from seven individuals (*Lanes 1–7*) are shown. The measured OPN levels of these samples by ELISA were 254, 699, 77, 79, 244, 120, and 98 ng/ml, respectively. *B*, linear regression analysis of the relationship between the measurement of OPN by ELISA and volume densitometry. Pearson correlation coefficient of the regression line was 0.83 ($P < 8 \times 10^{-8}$) for 16 representative plasma samples including those samples shown in *A*.

DISCUSSION

We have demonstrated that plasma OPN is significantly higher in ~70% patients with metastatic breast cancer ($n = 70$) compared with patients who are on clinical follow-up after completing all adjuvant therapy ($n = 44$) for early-stage breast cancer or healthy volunteers ($n = 35$). These results are consistent with the suggestion of Senger *et al.* (5) that plasma OPN levels may be elevated in metastatic disease. Furthermore, this is the first study to demonstrate significantly shorter survival for patients with metastatic breast cancer with increasing plasma OPN levels. The survival curves were statistically different when patients were grouped in terciles for plasma OPN ($P < 0.001$). Similarly, when OPN was treated as a continuous variable in a Cox proportional hazards model, there was a strong association between increasing OPN levels and decreasing survival. Graphical analysis of Martingale residuals showed no evidence of a threshold effect. The median survival was ~650 (extrapolated), 420, and 170 days for OPN values in the lower, middle, and upper thirds, respectively. In addition, we found an association between higher median plasma OPN levels and number of involved metastatic disease sites. Because patients with multiple metastatic sites would be expected to have a higher tumor burden than patients with only a single affected site, this would suggest that plasma OPN levels are an indicator of extent of disease. This is also suggested by the correlation of high plasma OPN with biochemical and hematological indicators believed to reflect poor prognosis, such as elevated serum alkaline phosphatase and AST, and low serum albumin and hemoglobin.

Because patient prognosis is largely related to tumor burden, a plasma assay that is reflective of extent of disease could be of great potential clinical utility. Presently, tumor burden is estimated clinically by a combination of physical findings and performance status, radiological tests, and hematological and biochemical parameters (such as bone marrow involvement, coagulopathy, and abnormal liver enzymes). None of these, in isolation, is sensitive enough to be used to monitor extent of disease or effectiveness of therapy. The need for such an assay becomes critical in clinical assessment regarding response to treatment, and hence in decision-making regarding continuation or the need to instigate change in therapy.

To this end, there has been a search for valid reproducible serum/plasma markers that could be used as indicators of extent of disease and response to treatment, not only for breast carcinomas, but for other cancers as well. There has been some success with regards to specific tumors [*e.g.*, CA 125 in the case of ovarian carcinoma (17–19), HCG and α-fetoprotein in the case of nonseminomatous germ cell tumors (20), PSA for prostatic carcinoma (21), CEA for colonic carcinoma (22), and serum hormone levels in various endocrine neoplasms]. In the case of breast carcinoma, a number of potential serum markers are presently undergoing evaluation [*e.g.*, CA 15.3 (23–25), mucin-like carcinoma-associated antigen and CA-549 (26–29), mucin-related antigens CAM 26 and 29 (30), CEA (31), and hepatocyte growth factor (32)]. However, none of these markers has been proven to satisfy the criteria necessary for routine use in clinical monitoring of the majority of patients with metastatic breast cancer.

OPN shows promise in this regard, because it is elevated in the majority of patients (at least 70%) with metastatic disease, appears to vary with tumor burden (as measured by number of metastatic sites in this study), shows little intra-individual variability in level upon repeated sampling in healthy women (15), and is readily measured in plasma by our recently developed ELISA assay (15). This assay depends on the epitope specificity of mAb53 (15), and our levels of OPN may reflect the availability and exposure of this epitope rather than the concentration of OPN. In addition, by its nature as a secreted phosphoprotein whose level of expression is apparently increased in breast cancer (6–8), OPN would have an immediate advantage over those molecules that are not biologically secreted into the extracellular milieu.

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